Legume chemistry and the specificity of the pea aphid (*Acyrthosiphon pisum*) host races

Dissertation

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“In every walk with nature one receives far more than he seeks.”

John Mui

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Summary

The pea aphid (*Acyrthosiphon pisum*) is a complex of at least 15 genetically different host races that are native to specific legume plants but can all develop on the universal host plant *Vicia faba*. Despite much research, it is still unclear why pea aphid host races can colonize their native host plants and *V. faba*, but not other legumes. After settling on the plant, all aphids penetrate the plant tissues with their stylets to reach the phloem sap rich in sugar and essential amino acids. During this path, aphids release effectors in their saliva that could generate specific plant chemical responses to the various pea aphid host races. Whether these responses are associated with host plant discrimination and acceptance by the pea aphids is still unknown. To determine the changes in the host plant chemistry and their possible influence on the specificity of the pea aphid host races, we used a full factorial experimental design separately infesting four different plant species (*Medicago sativa*, *Trifolium pratense*, *Pisum sativum*, and *Vicia faba*) with native and non-native pea aphid clones of various host races.

To find out whether legume species vary in their defense responses to different pea aphid host races, we measured the amounts of defense hormones in the four different plant species after infestation with native and non-native pea aphid clones. Additionally, we assessed the performance of the clones on the four plant species. On *M. sativa* and *T. pratense*, non-native clones that were barely able to survive or reproduce triggered a strong salicylic acid (SA) and jasmonate-isoleucine (JA-Ile) response, whereas infestation with native clones led to lower levels of both phytohormones. On *P. sativum*, non-native clones that survived or reproduced to a certain extent, induced fluctuating SA and JA-Ile levels, whereas the native clone triggered only a weak SA and JA-Ile response. The levels of the active JA-Ile conjugate generally corresponded well with the levels of the other JA pathway metabolites measured, suggesting that the reduction in JA signaling was due to an effect upstream of OPDA.

We searched for differences among the metabolomes of pea aphid host plant species and identified chemical compounds that change in an aphid host race-plant combination specific manner since these might be involved in pea aphid-host plant specialization. We used mass spectrometry-based non-targeted analyses of uninfested plants to reveal the existence of chemical differences between the investigated plant species. The most abundant unique

chemical compounds found in *M. sativa* and *T. pratense* belonged to saponins and flavonoids, respectively. Unique flavonoids were also found in *P. sativum* and *V. faba*. Additionally, a comparison of plant metabolic profiles from all aphid host race-plant species combinations revealed that the profiles of *M. sativa* and *T. pratense* plants infested with their respective native aphid host races were consistently different not only from those of uninfested control plants but also from profiles after the infestations with non-native host races. We tentative identified not only potential detrimental metabolites down-regulated exclusively by the native aphid host races, e.g., a glycosylated triterpene saponin with *m/z* 1086.55, but also potential defense metabolites induced only by the non-native host races, e.g., a flavonoid with *m/z* 695.30.

To identify the volatile compounds from the host plant species that might contribute to host selection by the pea aphid host races, we used headspace sampling combined with non-targeted mass spectrometry. The comparative analysis of the volatile profiles of the uninfested legume plants revealed that each species emit a particular blend. For example, *M. sativa* and *P. sativum* contained much higher amounts of methyl salicylate than the other plant species, and *T. pratense* contained significantly higher levels of (*E*)-β-farnesene. Furthermore, the pea aphid host races caused distinct changes also in the volatiles profiles of their host plants. At an interval of 48 hours after aphid infestation the green leaf volatiles (GLVs) *n*-hexanal, (*Z*)-3-hexenal, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate, were the most discriminating compounds among the aphid host race-plant combinations. The results revealed again that the native pea aphid host races reduced the emission of GLVs on their host plants more than non-native host races.

The combination of non-targeted and targeted metabolomic approaches proved a successful strategy to search for plant metabolites potentially involved in the acceptance of legume hosts by the various pea aphid host races. Although additional experimentation is necessary to fully understand the effects and influence of these compounds on host race specificity. These races have clearly evolved different feeding strategies to manipulate host plant defense signaling, reduce plant deterrence and toxicity, reduce enemies, and improve host quality.

Zusammenfassung

Die Erbsen-Blattlaus (*Acyrthosiphon pisum*) besteht aus mindestens 15 genetisch verschiedene Wirtsrassen, welche jeweils nur auf spezifischen Leguminosen natürlich vorkommen. Allerdings können sich alle Wirtsrassen auf der universellen Wirtspflanze *Vicia faba* entwickeln. Trotz intensiver Forschung ist bisher nicht bekannt, warum die Wirtsrassen der Erbsen-Blattlaus nur ihre spezifischen Wirtspflanzen und *V. faba* kolonisieren können. Wenn Blattläuse Pflanzen besiedeln, penetrieren sie das Pflanzengewebe mit ihren Saugrüsseln, um den an Zuckern und Aminosäuren reichen Phloemsaft zu erreichen. Während dieses Vorgangs werden Effektoren, welche spezifische chemische Antworten in den Pflanzen auslösen könnten, aus dem Speichel der Blattläuse freigesetzt. Ob diese Pflanzenantwort mit der Erkennung und Akzeptanz der Wirtspflanzen durch die Blattläuse zusammenhängt, ist bisher unbekannt. Um die chemischen Veränderung in den Wirtspflanzen und deren mögliche Einflüsse auf die einzelnen Blattlaus-Wirtsrassen zu bestimmen, haben wir einen vollfaktoriellen experimentellen Ansatz genutzt. Vier verschiedene Pflanzenarten (*Medicago sativa*, *Trifolium pratense*, *Pisum sativum*, and *Vicia faba*) wurden jeweils sowohl mit der jeweilig nativen als auch den nicht angepassten Blattlaus-Wirtsrassen besiedelt.

Um herauszufinden, ob die Abwehrantworten der Leguminosenarten auf unterschiedliche Blattlaus-Wirtsrassen variieren, haben wir die Phytohormonmengen in den vier Pflanzenarten nach Befall mit nativen und nicht angepassten Blattlaus Klonen und in unbefallenen Pflanzen gemessen. Zusätzlich haben wir die Entwicklung der Klone auf den vier verschiedenen Pflanzenarten bestimmt. In *M. sativa* und *T. pratense* haben nicht angepasste Klone, welche kaum in der Lage waren zu überleben oder sich zu reproduzieren, eine starke Salicylsäure- (SA) und Jasmonsäure-Isoleucin- (JA-Ile) Antwort hervorgerufen. Die Besiedlung mit nativen Klonen führte jedoch zur Bildung von nur geringen Mengen beider Phytohormone. Auf *P. sativum* induzierten nicht angepasste Klone, welche nur zu einem gewissen Maß überlebten und sich vermehrten, zu fluktuierenden SA und JA-Ile Gehalten. Der native Klon löste hingegen nur eine schwache SA und JA-Ile Antwort aus. Die Gehalte des aktiven JA-Ile Konjugates korrespondierten im Allgemeinen gut mit Gehalten anderer gemessener JA – Metabolite, was darauf hinweist, dass die Änderung im JA Signalweg vor der Bildung von OPDA erfolgte.

Wir suchten Unterschiede in den Metabolomen der Wirtspflanzenarten und identifizierten chemische Verbindungen, welche sich in Abhängigkeit von der Blattlaus Wirtsrasse – Pflanzen Kombination ändern, und dementsprechend bei der Blattlaus-Wirtspflanzen Spezialisierung eine Rolle spielen könnten. Wir nutzten einen nicht-gezielten massenspektrometrischen Metabolomik-Ansatz, um chemische Unterschiede zwischen den Pflanzenarten zu ermitteln. Die für *M. sativa* und *T. pratense* spezifischen Verbindungen, welche am häufigsten in diesen beiden Pflanzen gefunden wurden, gehörten zu den Saponinen und Flavonoiden. Pflanzenspezifische Flavonoide wurden auch in *P. sativum* und *V. faba* gefunden. Außerdem zeigte ein Vergleich der metabolischen Profile der mit unterschiedlichen Blattlaus-Wirtsrassen befallenen Pflanzen, dass sich die Profile von *M. sativa* und *T. pratense* nicht nur zwischen Pflanzen unterscheiden, die nicht mit Blattläusen bzw. mit dem jeweiligen angepassten Blattlausklon befallen sind, sondern auch zwischen Pflanzen, die mit dem nativen und den nicht angepassten Blattlausklonen befallen waren. Wir führten eine vorläufige Identifizierung von möglicherweise schädlichen Metaboliten durch, welche zum einen nur durch die nativen Blattlaus-Wirtsrassen herunter reguliert wurden, wie zum Beispiel ein glykosiliertes Triterpen Saponin mit m/z 1086.55, zum anderen durch die nicht angepassten Wirtsrassen induziert wurden, wie zum Biespiel ein Flavonoid mit m/z 695.30.

Um die flüchtigen Verbindungen der Wirtspflanzenspezies, die zur Wirtsauswahl durch die Blattlaus Wirtsrassen beitragen könnten, zu identifizieren, haben wir eine Duftsammlung mit einem nicht-gezielten massenspektrometrischen Metabolomik-Ansatz kombiniert. Die vergleichende Analyse der Duftstoffe von nicht mit Blattläusen befallenen Leguminosen ergab, dass jede Pflanzenart ein spezifisches Profil aufweist. Zum Beispiel enthielten *M. sativa* und *P. sativum* viel höhere Mengen von Methylsalicylat als die anderen Pflanzenspezies, während *T. pratense* signifikant höhere Level an (*E*)-β-Farnesen als *M. sativa* und *P. sativum* abgab. Desweiteren verursachten die Blattlaus-Wirtsrassen auch distinkte Änderungen der flüchtigen Verbindungen ihrer Wirtspflanzen. 48 h nach Blattlausbefall waren die grünen Blattduftstoffe *n*-Hexanal, (*Z*)-3-Hexenal, (*Z*)-3-Hexen-1-ol und (*Z*)-3-Hexenylacetat in *M. sativa* und *T. pratense* die am stärksten diskriminierenden Verbindungen. Die Ergebnisse bestätigten, dass native Blattlaus-Wirtsrassen die Abgabe von günen Blattduftstoffen stärker reduzieren als nicht angepasste Wirtsrassen.

Die Kombination von nicht gezielten und gezielten Metabolomik-Ansätzen erwies sich als erfolgreiche Strategie für die Suche nach pflanzlichen Metaboliten, welche möglicherweise für die Akzeptanz von Leguminosenarten als Wirte für verschiedene Blattlaus-Wirtsrassen verantwortlich sind. Allerdings sind weitere Untersuchungen nötig, um die Effekte und Einflüsse dieser Stoffe auf die Wirtsrassenspezifität zu verstehen. Die Wirtsrassen haben unterschiedliche Strategien entwickelt, mit denen sie die Signalwege der Wirtspflanzenabwehr manipulieren, die Abschreckung und Toxizität vermindern, die Anzahl der Feinde reduzieren, und somit die Qualität der Wirtspflanze erhöhen

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1 General Introduction

In their natural environments, plants have to confront multiple herbivores and pathogens. An extensive armor of structural and chemical-based defenses has evolved to protect against these attackers. These defenses are typically classified as constitutive or inducible (Walling, 2000). Constitutive defenses include physical barriers that block feeding or penetration, such as cell walls, spines, callose, waxy epidermal cuticles, and trichomes loaded with allelochemicals with adverse effect against insects (Walling, 2000;Gols, 2014). When plants detect the presence of attackers, inducible defenses are activated (Arimura et al., 2005;Pieterse et al., 2009). Detection is known to occur in two possible ways. First, enemies can be recognized by specific conserved components, known as herbivore-, microbe- or pathogen-associated molecular patterns (HAMPs/MAMPs/PAMPs). Pattern molecules are recognized by surface pattern-recognition receptors (PRRs) leading to pattern-triggered immunity (PTI). Second, herbivores and pathogens inject defense-suppressing effectors into the plant. However, plants have also evolved resistance (R) genes that encode receptors that recognize pathogen effectors, resulting in effector-triggered immunity (ETI) (Bent and Mackey, 2007;Pieterse et al., 2009;Wu and Baldwin, 2010;Robert-Seilaniantz et al., 2011;Santamaria et al., 2013;Lazebnik et al., 2014).

Plant attackers can also be differentiated into two groups depending on their behavior and feeding strategies. The first group includes organisms that actively destroy plant tissues, such as chewing-biting herbivores and necrotrophic pathogens. Chewing insects, for example, sever plant tissues and confront the constitutive defenses of the host. Then, their HAMPs or damage-associated molecular patterns (DAMPs) from the plants themselves are detected in plant tissue (Bonaventure, 2012). These pattern molecules induce PTI responses and in most cases activation of jasmonate (JA)-triggered defense responses. The second group of attackers includes organisms that require living hosts to obtain nutrients, such as piercing-sucking insects and biotrophic pathogens. In contrast to the first group, these attackers synthesize and release effectors in the plant tissues capable of preventing the induction of PTI. Nevertheless, plants often resist the attack of piercing-sucking herbivores and biotrophic pathogens via R genes and activation of ETI, which is usually associated with salicylic acid (SA)-triggered defense responses (Schweiger et al., 2014;Bigeard et al., 2015;Maag et al., 2015). Piercing-sucking

arthropods feed on cell content (mirids, spider mites), the xylem (spittlebugs, planthoppers, cicadas) and the phloem (whiteflies and aphids). Aphids are a large and well-studied group of piercing-sucking insect herbivores that feed on a large variety of commercially valuable crop plants and serve as vectors of around 50% of all plant viruses transmitted by insects (Kamphuis et al., 2013).

1.1 The pea aphid, *Acyrthosiphon pisum*.

One of the best-studied aphid species is the pea aphid *Acyrthosiphon pisum* (Figure 1). Whose genome was the first to be entirely sequenced among hemipterans (The International Aphid Genomics Consortium, 2010), and now is considered as a model for the study of ecological speciation (Brisson and Stern, 2006;Peccoud and Simon, 2010). The pea aphid is a legume specialist feeding on crops like lentil, bean, pea, alfalfa, and clover, as well as wild legume species. About 6200 years ago it underwent a rapid diversification, which led to the development of at least 15 different sympatric host races or biotypes specialized on specific host plants (Ferrari et al., 2006;Ferrari et al., 2008;Peccoud et al., 2009a;Peccoud et al., 2009b;Peccoud et al., 2015). Specialization on host plants is manifested by higher performance of pea aphid host races on their native hosts than on non-host plants. Such host plant preferences lead to assortative mating and reproductive isolation among populations (Del Campo et al., 2003). However, all pea aphid host races can perform well on *Vicia faba*, a universal host plant for all pea aphid biotypes characterized to date. Despite all of our existing knowledge on the pea aphid and its host races, the chemical mechanisms that are involved in host selection and host tolerance are virtually unknown.

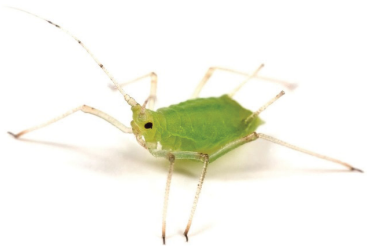

Figure 1. The pea aphid, *A. pisum*. By Ed Phillips. Stock Photo ID: 78005923 <https://www.shutterstock.com/image-photo/greenfly-aphid-on-white-wingless-form 78005923?src=PLkWoVvua4sWEOI8IqhBqQ-1-78>

1.2 Plant-aphid interactions

When aphids land on a plant, they may face many barriers to establish feeding (Goggin, 2007;Smith and Boyko, 2007;Howe and Jander, 2008;Wu and Baldwin, 2010). In most cases, plants will recognize new aphid colonizers either via HAMPs or DAMPs that lead to PTI (Hogenhout and Bos, 2011;Kaloshian and Walling, 2016), or by the detection of an effector in aphid saliva leading to an ETI (Santamaria et al., 2013;Lazebnik et al., 2014). Among all the molecules found in aphid saliva, protein effectors have been the most studied. De Vos and Jander (2009) found a 3-10 kDa protein from *Myzus persicae* that can induce defense responses in *Arabidopsis thaliana*. The defense responses triggered by *M. persicae* salivary effectors on *A. thaliana,* and *Nicotiana tabacum* have been shown to be detrimental to aphids reducing their fecundity (Bos et al., 2010;Elzinga et al., 2014). Protein effectors can induce an influx of Ca^{2+} ions, an important second messenger in signaling actions in plant cells (Wu and Baldwin, 2010). Ca^{2+} ions are associated with the production of reactive oxygen species (ROS) and other defense responses (Chen et al., 1993;Mai et al., 2013;Herrera-Vásquez et al., 2015).

The defense responses to aphids include the elicitation of the plant signaling pathways involving jasmonic acid-isoleucine (JA-Ile) and salicylic acid (SA) (Gao et al., 2007), which in turn promote the biosynthesis of a range of polar and non-polar, and volatile and non-volatile secondary metabolites of high chemical diversity (Wurtzel and Kutchan, 2016). Some of these compounds can be involved in direct plant defenses against aphids as toxic, repellent, or antinutritive molecules, while others can serve as signals in indirect defense for the attraction of aphid predators, (Wink, 2003;Leitner et al., 2005;Mazid et al., 2011;Bednarek, 2012). Over the course of evolution, aphids have long had to cope with the existence of these defenses and have developed adaptations that allowed them to feed and specialize on specific host plants (Ehrlich and Raven, 1964;Berenbaum and Zangerl, 1998).

The chemistry of legume host plants is thought to play a decisive role in the initiation and maintenance of aphid feeding and subsequent growth and survival (Del Campo et al., 2003). Before choosing a suitable plant for feeding, pea aphids may confront a blend of plant volatile compounds that might be used as cues to attract them to their host plants, but also could be repellent or deterrent (Kamphuis et al., 2013). Once an aphid is on a plant, they insert their stylets into intercellular spaces to reach the phloem sieve elements. During this process, aphids

collect gustatory information thanks to repetitive punctures of plant cells, which help them decide whether to continue towards the phloem or withdraw and begin a new stylet insertion in a new location. Thus, encounters with different metabolites, both nutrients and defenses could be a primary factor for controlling the initiation of stylet insertion and continued penetration (Powell et al., 2006;Pompon and Pelletier, 2012;Schwarzkopf et al., 2013).

1.3 Questions addressed in this thesis

Researchers have long sought to understand why the various pea aphid host races have a high specificity for their host plants. For example, one study identified plant tissues containing factors controlling host specificity (Schwarzkopf et al., 2013). This work employed three different pea aphid host races, native to, *Medicago sativa* (alfalfa), *Pisum sativum* (pea), and *Trifolium pratense* (red clover), respectively, and four plant species, *M. sativa*, *P. sativum*, *T. pratense*, and *Vicia faba* (broad bean), accepted as a host by all of the races. Although some attempts have been made to elucidate the role of legume chemistry in this plant-herbivore system (Schwartzberg et al., 2011;Goławska et al., 2014;Morkunas et al., 2016;Hopkins et al., 2017), we still have little knowledge of whether chemical compounds control host choice and performance.

The objective of this research work was to determine the chemical basis of pea aphid-plant interactions between four plant species and three aphid host races. The plants (*M. sativa*, *P. sativum*, *T. pratense* and *V. faba*) are all agriculturally and ecologically important legumes, and the host races included one each native to *M. sativa*, *P. sativum,* and *T. pratense*, with all able to feed on the broad bean. First, we determined the performance of each host race over a four-day time period feeding on each of the plant species (Chapter No.1). Second, we investigated the effects of infestations with each pea aphid host race on the defense hormones of each plant species and how aphids might manipulate hormone signaling by interfering with specific biosynthetic steps of the JA pathway (Chapter No. 1). Hormone analysis was carried out via a targeted mass spectrometry-based metabolomic protocol. Third, we used a non-targeted mass spectrometry-based metabolomic approach with plant polar and semi-polar fractions to identify the chemical differences among plant species and determine the effect that aphid host race infestation had on the levels of these compounds (Chapter No. 2). The results should point to metabolites responsible for the choice and acceptance of host plants by the host

races. Fourth, we used a combination of targeted and non-targeted metabolomic approaches to determine the differences in the volatile profiles of the legume species and the changes caused by aphid host race infestation with emphasis on changes in green-leaf volatiles (GLVs).

2 Research Chapter 1. Modulation of Legume Defense Signaling Pathways by Native and Non-native Pea Aphid Clones

This chapter is a partially adapted version of our paper written by Sanchez-Arcos, C., Reichelt, M., Gershenzon, J., and Kunert, G. (2016). Titled: Modulation of Legume Defense Signaling Pathways by Native and Non-native Pea Aphid Clones, and published in Frontiers in Plant Science 7 (Sanchez-Arcos et al., 2016).

2.1 Introduction

The best studied reactions in plants are the biosynthesis of phytohormones involved in the signal transduction pathways (Mauch-Mani and Mauch, 2005;Pieterse et al., 2009;Cao et al., 2011;Morkunas et al., 2011;Pieterse et al., 2012;Denancé et al., 2013;Wasternack and Hause, 2013;Caarls et al., 2015), among which salicylic acid (SA) and jasmonic acid-isoleucine (JA-Ile) are the two primary and well known defense-related compounds. While the SA defense pathway has mainly been associated with the response against biotrophic pathogens, the jasmonic acid (JA) defense pathway affects herbivorous insects and necrotrophic pathogens (Pieterse et al., 2012), and is mainly activated after wounding (Howe, 2004). Both defense pathways are, however, strongly crosslinked (De Vos et al., 2005;Beckers and Spoel, 2006;Koornneef and Pieterse, 2008;Pieterse et al., 2009;Gimenez-Ibanez and Solano, 2013;Caarls et al., 2015). SA can negatively affect JA signaling downstream of the SCF^{COI1}-JAZ complex (Koornneef et al., 2008;Zhang et al., 2009;Van der Does et al., 2013;Zhang et al., 2013), and that JA can suppress the SA defense pathway (Brooks et al., 2005;Nomura et al., 2005). This crosstalk between SA and JA signaling could be synergistic (Schenk et al., 2000;Mur et al., 2006), but is the timing and the sequence of SA and JA signaling initiation (Koornneef et al., 2008;Leon-Reyes et al., 2010) as well as the levels of phytohormones that seem to be important for specific defense responses (Mur et al., 2006). Other phytohormones like abscisic acid (ABA) play an important role in fine-tuning of the defense responses of the plants interfering with JA and SA signaling (Mauch-Mani and Mauch, 2005;Ton et al., 2009;Cutler et al., 2010;Cao et al., 2011;Morkunas et al., 2011;Denancé et al., 2013). ABA promotes early defense responses by closing stomata and stimulating callose deposition, which blocks the intrusion of the pathogen into plant tissue. In late responses, ABA could interact with

other defense pathways inhibiting the SA-dependent responses or modulating the JA-dependent pathway (Yasuda et al., 2008;Ton et al., 2009;Pieterse et al., 2012;Finkelstein, 2013). Although there is still much to learn about the regulation of hormonal crosstalk, it is assumed that these mechanisms provide plants with an adaptable system capable of tuning defense responses specific to different classes of attackers (Pieterse et al., 2012) and resulting in the synthesis of toxic or deterrent defense compounds that prevent the colonization of the plant.

Aphids can employ a range of strategies to overcome plant defenses (Walling, 2008;Giordanengo et al., 2010;Kamphuis et al., 2013;Will et al., 2013;Jaouannet et al., 2014). They may be able to detoxify defense harmful compounds, induce the nutritional capacity of the host. Besides these strategies, effector proteins in aphid saliva may also hinder activation of plant defenses and so may decrease phytohormone signaling. For example, Mp55, an effector molecule from *M. persicae* suppressed the formation of three defense compounds in *A. thaliana*: 4-methoxyindol-3-ylmethyl glucosinolate, callose, and hydrogen peroxide (Elzinga et al., 2014). A structural protein of the stylet sheath, necessary for sealing the stylet penetration site, might prevent the influx of Ca^{2+} ions and the activation of Ca^{2+}-dependent defense signaling machinery (Abdellatef et al., 2015;Furch et al., 2015). Calcium binding proteins in aphid saliva seem to have the same effect (Will et al., 2007). In other cases, the mode of action of salivary effectors like Armet and C002 from *A. pisum* (Mutti et al., 2006;Mutti et al., 2008;Wang et al., 2015), Me10 and Me23 from the potato aphid *Macrosiphum euphorbiae*, and PIntO1 and PIntO2 from *M. persicae* (Pitino and Hogenhout, 2013) is not known, but the evidence in these studies showed that they enhanced the performance of the aphids on the respective host plants. In contrast, silencing the encoding genes of these protein effectors by RNAi reduced aphid fecundity (Mutti et al., 2006;Mutti et al., 2008;Bos et al., 2010;Pitino et al., 2011). Therefore, these proteins may also interfere with defense signaling pathways and alter phytohormone biosynthesis. Thus the measurement of phytohormone levels after aphid infestation may provide excellent indications about whether these insects trigger or block defense signaling on different host plants.

There have been attempts to find the genomic regions associated with plant adaptation of pea aphid host races (Hawthorne and Via, 2001;Jaquiery et al., 2012;Simon et al., 2015). A genome-wide study of pea aphid host races was conducted and a few loci encoding salivary proteins were identified in regions under putative divergent selection (Jaquiery et al., 2012).

Previous investigations in our group about the feeding behavior revealed that regardless of whether they are on their native host plant or another legume species pea aphids start to penetrate the plant and to pierce and salivate into plant cells (Schwarzkopf et al., 2013). In order to find out what is salivated into the plant, transcriptomic analysis of salivary glands was conducted and around 600 pea aphid salivary genes described (Carolan et al., 2011) and proteins were identified by proteomic analysis of saliva (collected from artificial diet fed by aphids) or salivary glands (Carolan et al., 2009;Carolan et al., 2011;Vandermoten et al., 2014). Several studies concluded that these salivary proteins might suppress plant defense responses in native host plants (Mutti et al., 2008;Pitino and Hogenhout, 2013) or trigger defense reactions in non-host plants (Li et al., 2006;Will et al., 2007;Gao et al., 2008;Hogenhout and Bos, 2011). To confirm the capacity of these salivary protein effectors would be useful to determine how are the phytohormone levels responses among various host plant species-aphid host race combinations.

To find out why the pea aphid host races can perform well on their native or the universal host plant while they are not able to colonize other plants, an important step would be to measure the defense phytohormone levels to determine whether defenses are being activated or not. The detection of differences in phytohormone levels modulated by native vs. non-native host races would favor the hypothesis that native aphid races are able to manipulate plant defense activation processes for their benefit. So far, there is just one study investigating the phytohormone response of a native host plant (*Pisum sativum*) to pea aphid infestation, but concentrated on changes due to aphid numbers and only used an aphid clone that was native to *P. sativum*.(Mai et al., 2014). Thus information about how pea aphid host plants react to non-native pea aphid host races is still lacking. Therefore, in this study, we investigated the phytohormone response of three native host plants of the pea aphid, *M. sativa*, *P. sativum*, *T. pratense*, and the universal host *V. faba* over a four-day time course after infestation with native and non-native aphid clones for each plant species. We analyzed levels of the jasmonic acid-isoleucine conjugate (JA-Ile), salicylic acid (SA) and abscisic acid (ABA), and also quantified several other jasmonates to explore how aphids might manipulate hormone signaling by interfering with specific biosynthetic steps. Also, we determined the performance of native and non-native aphid host races on each plant species over the same time period. Although data are available in the literature on pea aphid reproduction on different hosts, this information is for

plants of different ages and varieties and from different growing conditions than used here and did not assess the survival and growth of adult aphids.

2.2 Materials and methods

2.2.1 Plant material

Four legume plant species: *Medicago sativa* cultivar (cv.) 'Giulia' (alfalfa), *Trifolium pratense* cv. 'Dajana' (red clover), *Pisum sativum* cv. 'Baccara' (pea), and *Vicia faba* cv. 'The Sutton' (broad bean), were grown in 7-cm diameter plastic pots with a standardized soil mixture (7:20 mixture of Klasmann Tonsubstrat and Klasmann Kultursubstrat TS1, Klasmann-Deilmann GmbH, Geeste, Germany) in climate chambers maintained at 20 °C, 70 ± 10% relative humidity, and 16 h light/8 h dark photoperiod. *M. sativa* and *T. pratense* were grown three plants per pot to get enough plant material for phytohormone analyses (approximately ten and six leaves per pot, respectively), while *P. sativum* and *V. faba* were grown individually grown individually (approximately four leaves per pot for each species). *M. sativa* and *T. pratense* plants were used in experiments 20 days after sowing and *P. sativum* and *V. faba* 10 days after sowing.

2.2.2 Aphids

Three pea aphid (*Acyrthosiphon pisum* Harris) clones, each representing one pea aphid host race, were used in the experiments: the clone L84 representing the *Medicago* race (here called MR), the clone T3-8V1 representing the *Trifolium* race (TR), and the clone Colmar representing the *Pisum* race (PR). Aphids were initially collected from their native host plants *T. pratense*, *M. sativa,* and *P. sativum*, respectively, and genotypically assigned to their respective host race (for detailed information see Table S1 in Peccoud (2009a)). All aphids were reared on 4-week-old broad bean plants. To synchronize the age of the aphids for the experiments, five apterous female adults were placed on a broad bean plant and were allowed to reproduce for 48 h. The nymphs were then transferred to new plants and maintained for nine days until they reached the adult age. Several serial transfers of nymphs were done until the desired number of synchronized young adult aphids was obtained. To avoid escape of aphids,

all aphid containing plants were covered with air permeable cellophane bags (18.8 x 39 cm, Armin Zeller, Nachf. Schütz & Co, Langenthal, Switzerland), and placed in a climate chamber under the conditions described above.

2.2.3 Experimental design

To determine the performance of the three different pea aphid clones of various host races, each plant species was separately infested with each pea aphid clone resulting in 12 plant species–aphid clone combinations. To evaluate the development of the different pea aphid clones over time, plants were infested with 20 adults, apterous aphids, and performance parameters were measured 24, 48, 72, and 96 h after aphid infestation and at the start of the experiment. Survival and mean weight of adult aphids (weight of all alive adult aphids on a plant divided by the number of surviving adult aphids), and the weight of all offspring per plant were measured as performance parameters. To keep the aphids as undisturbed as possible (and to duplicate the setup used in the phytohormone experiment described below), different sets of plants and aphids were used at each time point. For this performance experiment, five replicates were used.

To evaluate the response of the plant species towards infestation with the different pea aphid clones, phytohormone levels were investigated. The experimental setup was the same as for the performance experiment with 12 plant species –aphid clone combinations sampled at four-time points. Additionally, plants without aphids served as controls. Ten replicates were employed.

All experimental plants, including aphid-free control plants, were covered with air permeable cellophane bags and were placed in a climate chamber under conditions as described above.

2.2.4 Plant material sampling and extraction

For plant sampling, the aphids were removed from the plants using a paintbrush. As a control for possible induction of phytohormones due to contact with the paintbrush, control plants were brushed in the same way as aphid-infested plants. Above ground parts of the plant seedlings were harvested and rapidly frozen in liquid nitrogen. Frozen samples were stored

overnight in two ml Eppendorf tubes at -80 °C and then freeze-dried for 48 h. Dried plant material was homogenized into a fine powder by adding three stainless steel beads (3 mm Ø) in each tube and vigorously shaking for four min in a paint shaker (Skandex shaker SO-10m, Fast & Fluid Management, Sassenheim, The Netherlands). Portions (10 mg) of dried plant material were extracted with 1 ml ice-cold extraction solution containing 80% methanol acidified with 0.1% formic acid with deuterated or ^{13}C-labeled phytohormones as internal standards, (40 ng ml^{-1} of jasmonic acid-d_6, salicylic acid-d_4, and abscisic acid-d_6, and eight ng ml^{-1} of jasmonic acid-$^{13}C_6$-isoleucine conjugate). Samples were immediately vortexed for 10s and continuously sonicated in a water bath at room temperature (20°C) for 15 min at maximum frequency (35 kHz). After centrifugation (10 min at 4,500 *g* and -10°C), supernatants were filtered using 0.45mm PTFE AcroPrep™ 96-well filtration plates (Pall Corporation, Port Washington, NY, USA) and a vacuum filtration unit. All filtered plant extracts were stored at -80 °C until LC-MS/MS analysis.

2.2.5 Quantification of phytohormones by LC-MS/MS

Chromatographic separation of phytohormones was performed on an Agilent 1260 High-performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA, USA). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 µm, Agilent). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B respectively. The elution profile was: 0-0.5 min, 10% B; 0.5-4.0 min, 10-90% B; 4.0-4.02 min, 90-100% B; 4.02-4.50min, 100% B, 4.50-4.51 min 100-10% B and 4.51-7.00, 10%B. The mobile phase flow rate was 1.1 ml/min. The column temperature was maintained at 25ºC. An API 5000 tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a Turbospray ion source was operated in negative ionization mode. The instrument parameters were optimized by infusion experiments with pure standards, where available. The ion spray voltage was maintained at -4500 eV. The turbo gas temperature was set at 700ºC. Nebulizing gas was set at 60 psi, curtain gas at 25 psi, the heating gas at 60 psi and collision gas at seven psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion → product ion fragmentations as follows: *m*/*z* 136.9 →93.0 (collision energy (CE)-22 V; declustering potential (DP) -35 V) for salicylic acid (SA); *m*/*z* 140.9 →97.0 (CE -22 V; DP -35 V) for SA-d_4; *m*/*z* 290.9 →165.1 (CE -24V; DP -45V) for 12-oxo phytodienoic acid (OPDA); *m*/*z* 209.1 →59.0 (CE -24 V; DP -35 V) for jasmonic acid (JA); *m*/*z* 215.1 →59.0 (CE -24 V;

DP -35 V) for JA-d_6; *m/z* 225.1 →59 (CE -24V; DP -35V) for the two hydroxyjasmonic acid isomers (here designated OH-JA1 and OH-JA2, respectively); *m/z* 322.2 →130.1 (CE -30V; DP -50V) for JA-isoleucine conjugate (JA-Ile); *m/z* 328.2 →136.1 (CE -30V; DP -50V) for JA-$^{13}C_6$-Ile; *m/z* 338.1 →130.1 (CE -30V; DP -50V) for 12-OH-JA-Ile; *m/z* 352.1 →130.1 (CE -30V; DP -50V) for 12-carboxyjasmonic acid-isoleucine conjugate (12-COOH-JA-Ile); *m/z* 263.0 →153.2 (CE -22 V; DP -35 V) for abscisic acid (ABA); *m/z* 269.0 →159.2 (CE -22 V; DP -35 V) for ABA-d_6. The hydroxyjasmonic acids include the 11- and 12-hydroxy derivatives (Miersch et al., 2008;Stitz et al., 2011), but we were unable to distinguish between them. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.6 software (Applied Biosystems) was used for data acquisition and processing. Linearity in ionization efficiencies was verified by analyzing dilution series of standard mixtures. Phytohormones were quantified relative to the signal of their corresponding internal standard. For quantification of OPDA and OH-JA, the internal standard JA-d_6 was used applying experimental response factors of 0.5 and 1.0 respectively. These response factors were determined by analyzing a mixture of OPDA and OH-JA (both kindly provided by W. Boland, MPI for Chemical Ecology, Jena, Germany; synthesized as described in (Nakamura et al., 2011;Shabab et al., 2014)) and JA-d_6 all at the same concentration. For OH-JA-Ile and COOH-JA-Ile quantification, JA-$^{13}C_6$-Ile was used as internal standard applying a response factor of 1.0 in both cases. The response factor for OH-JA-Ile was determined by analyzing a mixture of OH-JA-Ile (kindly provided by W. Boland, MPI for Chemical Ecology, Jena, Germany; synthesized as described in (Jimenez-Aleman et al., 2015)) and JA-$^{13}C_6$-Ile at the same concentration. The response factor for COOH-JA-Ile was assumed to be similar. All metabolite levels are expressed in nanograms per gram dry weight (ng g^{-1} DW).

2.2.6 Chemicals

The sources of the phytohormone standards were jasmonic acid-d_6 (HPC Standards GmbH, Cunnersdorf, Germany), salicylic acid-d_4 (Sigma-Aldrich), abscisic acid-d_6 (Santa Cruz Biotechnology, Dallas, TX, USA), and jasmonic acid-$^{13}C_6$-isoleucine conjugate (synthesized as described by Kramell et al. (1988) using $^{13}C_6$-Ile (Sigma-Aldrich)).

The sources of the solvents used for the phytohormone extraction were methanol (LiChrosolv®, LC-MS grade, Merck KGaA, Germany), acetonitrile (LC-MS grade, VWR Chemicals, USA), and formic acid (LC-MS grade, Fisher Scientific, Belgium).

2.2.7 Statistical analysis

All data were analyzed with R version 3.2.0 (R Development Core Team, 2015).

The percentage of surviving adults was analyzed using binomial generalized linear models (glm) with time after aphid infestation as continuous and aphid clone as categorical explanatory variables. In cases of overdispersion, standard errors were corrected using quasi-glm models. P-values for explanatory variables were obtained by deleting explanatory variables one after another and comparison of the most complex model with the simpler model (Zuur et al., 2009).

To make the progression of aphid weight over time comparable between the different aphid clones, the weight of surviving adult aphids is given as a percentage of the weight at the start of the experiment which was set as 100%. These data were analyzed using a two-way ANOVA with the time points and aphid clones as categorical explanatory variables. Models were simplified by deleting non-significant variables (Crawley, 2013). To determine differences between factor levels, pairwise t-tests were performed and corrected for the false discovery rate. In cases where variances were unequal, the generalized least squares method (gls from the nlme library (Pinheiro et al., 2015)) was used. First, the optimal variance structure was determined by comparing models with different variance structures and choosing the one with the smallest AIC (Akaike information criterion). Models with this variance structure were used to determine the influence of explanatory variables by subsequent removal of explanatory variables from the model and comparison of the simpler with the more complex model with a likelihood ratio test (Zuur et al., 2009). Differences between factor levels were determined by factor level reduction (Crawley, 2013).

The influence of the aphid clone and time on the offspring biomass was investigated with a two-way ANOVA. To achieve homogeneity of variances, biomass data were square root transformed. Differences between factor levels were examined by pairwise t-tests corrected for false discovery rate.

The influence of aphid clone and duration of aphid infestation (both used as categorical explanatory variables) on the phytohormone levels was investigated using the generalized least squares method (gls from the nlme library (Pinheiro et al., 2015)) to account for the variance heterogeneity of the residuals. The varIdent variance structure was used. Whether the different variance of aphid clones, the duration of aphid infestation or the combination of both factors should be incorporated into the model, was determined by comparing models with different variance structures with a likelihood ratio test and choosing the model with the smallest AIC. The influence (*p*-values) of the explanatory variables was determined as explained above in the analysis of adult weight.

2.3 Results

2.3.1 Aphid host race clones performed much better on their native host plants

The performance of pea aphid clones of various host races on the different plant species over time was determined by measuring the survival and weight of adult aphids, and the total weight of aphid offspring.

The survival over time of all aphid clones was significantly better in their respective native host plant and in the universal host plant *V. faba* than in the non-native host plants. Although the survival of all aphid clones on all host plants decreases over time, more than the 80% of native clones survived for four days (96h) on their host plants and on *V. faba* (Figure 2A-D; Table S1). On *M. sativa* the survival of the non-native aphids (TR and PR) drop steadily, having around half of the aphids dead by 48 h, and any survival by 96h (Figure 2A). On *T. pratense* the non-native *Medicago* clone (MR) showed intermediate survival with nearly 48% of the aphids alive at 96h in comparison with the low survival displayed by the non-native *Pisum* clone (PR) where only a few aphids survived at the same time (Figure 2B). On *P. sativum*, the non-native MR showed similar survival as the native PR, being the only case where the general pattern was not followed. On the other hand, the non-native *Trifolium* clone (TR) showed sharply reduced survival (Figure 2C). On the universal host plant *V. faba,* all aphid clones survived equally well (Figure 2D).

Surviving adult aphids of native clones on their host plants lost significantly less weight than non-native aphid clones feeding on the same plant species. Nonetheless, all surviving adult aphids lost weight significantly over time on all the plants (Figure 2E-H, Table S2). On *M. sativa* the weight percentage over time of the surviving aphids of both non-native clones (TR and PR) were significantly lower than the native MR aphids(Figure 2E). On *T. pratense* plants the tendency is more noticeable, with both non-native clones (MR and PR) losing about 60 % of their original weight, while the native TR lost about 20% of its initial weight over the course of the experiment (Figure 2F). On *P. sativum*, the weight percentage of surviving non-native TR aphids was lower over time than the ones measured for the non-native MR and the native PR aphids (Figure 2G). In contrast, on the universal host *V. faba*, surviving aphids of all clones showed a tendency to either kept their initial weight for the first two to three days or even gained weight. Only after this time did they start to lose weight (Figure 2H).

The maximum total weight of aphid offspring produced during the experiment was always observed for each aphid clone on their native host plant. The total weight of these native clones offspring incremented significantly over time and was more abundant than the amounts obtained from non-native aphid clones (Figure 2I-K, Table S3). On *M. sativa* non-native aphids clones TR and PR barely produced offspring in comparison with the higher amounts generated by the native clone MR (Figure 2I). Similarly, on *T. pratense* plants the non-native aphid clones PR and MR showed a lower total weight of offspring in comparison to the native aphid clone TR. However, the non-native MR on this plant displayed an intermediate production of offspring in comparison with the other aphid clones (Figure 2J). On *P. sativum* plants, the total weight of offspring significantly increased during the experiment for all the aphid clones, although the native aphid clone PR showed significantly higher production of offspring (Figure 2K). A similar tendency over time was observed for the total offspring weight of all the aphid clones on the universal host *V. faba*. However, on this plant, the TR was the clone that showed the higher production of offspring compared to the other aphid clones. The total offspring weight obtained in the universal host plant for all aphid clones was always more prominent than the total offspring weight observed in the other plant species (Figure 2L).

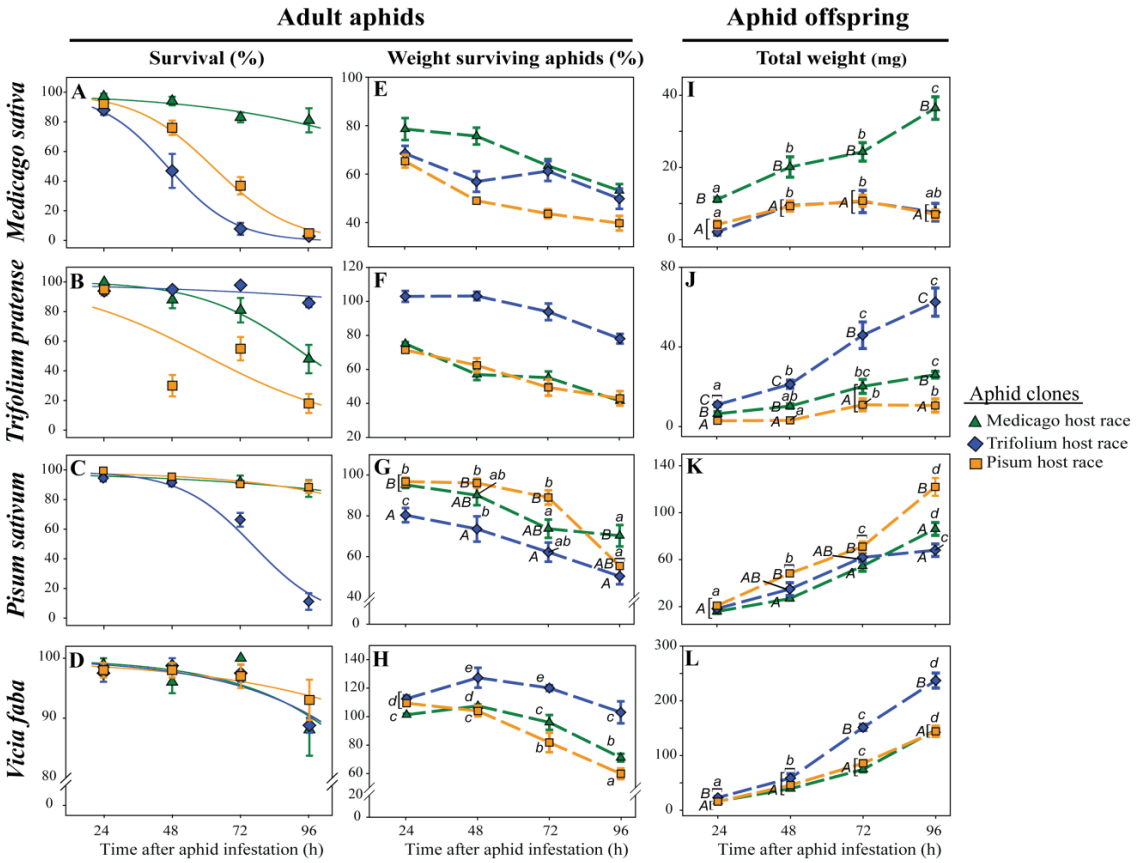

Figure 2. Performance of pea aphid clones of different host races on native and non-native legume species. Survival of adult aphids (A-D), mean weight of surviving adult aphids (E-H), and total weight of offspring (I-L) are depicted for three aphid clones tested on *M. sativa*, *T. pratense*, *P. sativum*, and *V. faba* plants and measured 24, 48, 72, and 96 hours after aphid infestation. The aphid clones represent the *Medicago*, *Trifolium* and *Pisum* host races. Symbols represent means ± SE. Statistical values are given in Tables 1 (A-D), 2 (E-H), and 3 (I-L). Only in cases where a significant influence of the aphid clone on the weight of the surviving adults or the total weight of the offspring was dependent on the time after aphid infestation (time x race interaction), post-hoc tests or similar methods were used to reveal differences between aphid clones at different time points. Different letters indicate significant differences ($P \leq 0.05$). For the sake of clarity, upper case letters in panels G, I, J, K, and L indicate significant differences between aphid clones within a specified time point, while lower case letters indicate significant differences between different time points within one aphid clone. (A-D) Solid lines in the survival graphs are the fitted curves from the generalized linear model (glm). (E-H) The mean weight of surviving adult aphids is given as a percentage of the weight at the start of the experiment which was set as 100%.

2.3.2 Clones of native host races induced lower levels of SA and JA-Ile than clones of non-native races

To determine how the clones of the different pea aphid host races affected the defense responses of the various plant species, we used a targeted metabolomic approach to quantified the levels of three plant hormones: salicylic acid (SA), jasmonic acid-isoleucine (JA-Ile), and abscisic acid (ABA), in each plant species separately infested with each of the aphid clones and in uninfested control plants.

The levels of SA in infested plants changed in an aphid-clone specific manner. In contrast, uninfested plant kept almost constant levels over time (Figure 3A-D, Table S4). In *M. sativa*, the levels of SA after the infestation with all aphid clones were always significantly higher than those levels measured in uninfested control plants. Moreover, infestations with non-native aphid PR and TR clones elicited significantly higher concentrations of SA than native MR clone for the first 72 hours. However, after this time the SA levels in plants infested with non-native clones decreased, while the levels in plants infested with native MR clone increased to significantly higher levels (Figure 3A). Similarly, in *T. pratense* concentrations of SA were significantly higher over time after infestation with non-native MR and PR clones than the levels estimated after the infestation with native TR clone and the constitutive concentrations measured in uninfested control plants. The levels of SA in plants infested with native TR clone fluctuated from higher to lower than levels in uninfested control plants (Figure 3B).

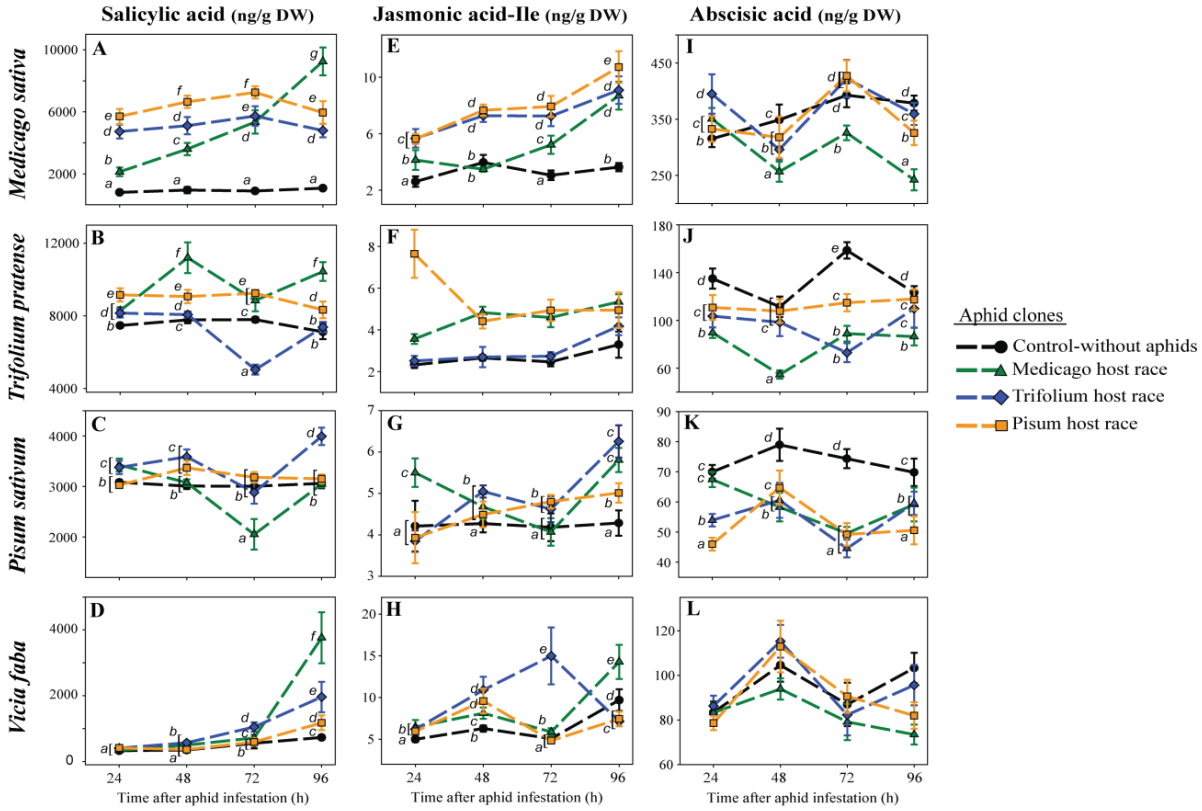

Figure 3. Levels of salicylic acid, jasmonic acid-isoleucine and abscisic acid in legume plants after infestation with pea aphid clones of different host races. Symbols represent means ± SE. Statistical values are presented in Table S4. In cases where a significant influence of the aphid clone on the phytohormone level was dependent on the time after aphid infestation (interaction), significant differences ($P \leq 0.05$) between aphid clones at different time points are indicated by different letters.

In *P. sativum*, the SA levels changed less over time, especially when plants were infested with the native PR clone, measured levels were equivalent to levels in uninfested control plants. On the other hand, the SA levels in plants infested with non-native MR and TR clones fluctuated over time without a specific pattern, and were higher (TR at all time points except 72 h, MR at 24 h), lower (MR at 72 h) or similar (MR at 48 h and 96 h, TR at 72 h) than those found in uninfested control plants (Figure 3C). In contrast, the measured SA concentrations did not change markedly during the first 72 hours in the universal host *V. faba* after the infestation with all the aphid clones. However, 96 hours after the infestation with the aphid clones, the levels of SA were significantly higher than in uninfested control plants. Similar than in *P. sativum*, PR was the clone that triggered the lowest changes in SA concentrations compared with levels elicited by the infestation with MR and TR clones (Figure 3D).

The variations of the concentrations of JA-Ile in infested plants are aphid clone dependent. Besides, the JA-Ile level in uninfested control plants showed a similar trend that SA levels, remaining stable over time with lower changes in comparison with those changes triggered by aphid infestation (Figure 3E-H, Table S4). In *M. sativa* and *T. pratense* during the first 72 hours of experiment, the JA-Ile concentrations were significantly higher in plants infested with the non-native clones (TR and PR for *M. sativa*; MR and PR for *T. pratense*) compared to levels measured in plant infested with the native clones (MR for *M. sativa*; TR for *T. pratense*) or uninfested control plants. While in *M. sativa* the infestation with the native MR clone elicited significantly higher concentrations of JA-Ile than the levels measured in uninfested control plants, in *T. pratense* the levels of JA-Ile in native clone- infested plants and uninfested control plants were in the same range. After 72 hours, in both plant species, the levels of JA-Ile of native aphid clones- infested plants incremented and reached similar levels as in plants infested with the non-native aphid clones (Figure 3E-F).

When *P. sativum* plants were infested with the native PR clone, the estimated JA-Ile levels raised from concentrations equivalent (24 hours) to significantly different (72-96 hours) than those measured in uninfested control plants, but still lower than in plants infested with non-native aphid clones (MR, TR). On the other hand, the SA-Ile levels in *P. sativum* plants infested with non-native clones fluctuated over time, being as low as in control plants (TR at 24 h, MR at 72 h) or significantly higher than in control plants (TR at 48 h, 72 h, and 96 h, MR

at 24 h, 48 h, and 96 h) (Figure 3G). In *V. faba* plants, during the first 48 hours, the JA-Ile concentrations slightly increased in all aphid-infested plants, always with higher levels than in control uninfested plants. After this time point, the JA-Ile concentrations over time are aphid clone dependent. At 96 hours after aphid infestation, JA-Ile levels in aphid-infested plants were lower (PR- and TR-infested plants), or higher (MR-infested plants) than in uninfested control plants (Figure 3H).

The ABA concentrations fluctuated over time in an aphid clone-dependent manner in all plant species but in *V. faba* (Figure 3I-L, Table S4). There were no differences between the ABA accumulation patterns of infestations with native and non-native aphid clones. ABA levels in aphid-infested plants were either lower or equivalent to levels determined in uninfested control plants (Figure 3I-L). Only in *M. sativa* at 24 hours after aphid infestation, ABA levels in aphid-infested plants were significantly higher than in uninfested control plants (Figure 3I).

2.3.3 Clones from native host races induced lower levels of JA pathway metabolites than non-native races

To obtain information about the effect of pea aphid clones infestation on the synthesis and metabolism of the active jasmonate, JA-Ile, we measured the levels of its precursors the 12-oxo phytodienoic acid (OPDA), and jasmonic acid (JA), as well as its metabolites, the 12-hydroxyjasmonic acid-isoleucine conjugate (12-OH-JA-Ile), the 12-carboxyjasmonic acid-isoleucine conjugate (12-COOH-JA-Ile), and two hydroxylated forms of unconjugated JA (OH-JA1 and OH-JA2).

In *M. sativa*, all determined JA-Ile precursors and structurally related derivatives were generally present in significantly lower levels after infestation with the native MR clone than after the non-native TR and PR clones (Figure 4, Table S4). This pattern was even more evident for the JA-Ile precursors: OPDA and JA (Figure 4A and B). The concentrations of the hydroxylated and carboxylated JA and JA-Ile related derivatives over time were mostly lower in plants infested with the native MR clone, and similar to levels measured in uninfested control plants, but increased after 72 hours reaching levels equivalent to those found in plants infested with non-native aphids TR and PR clones at 96 hours after aphid infestation (Figure 4E-G).

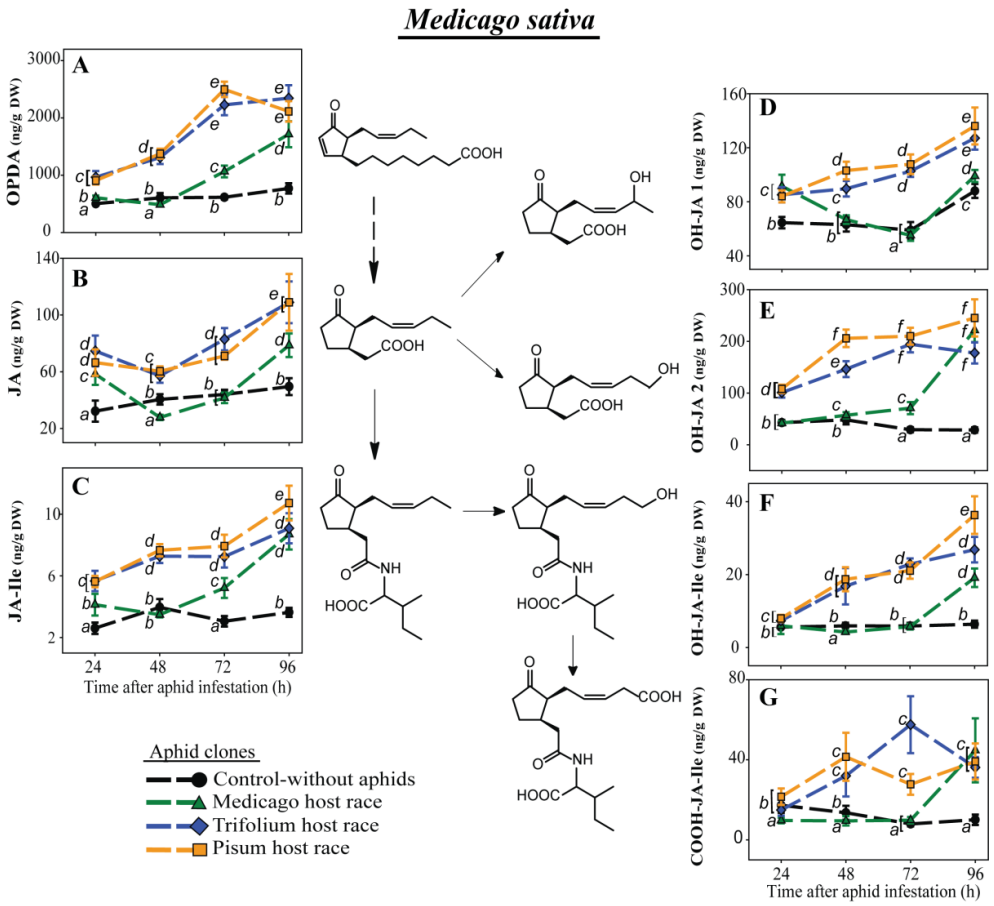

Figure 4. Level of JA pathway metabolites in *M. sativa* plants after infestation with pea aphid clones of different host races. Symbols represent means ± SE. Statistical values are presented in Table S4. Different letters indicate significant differences between treatments ($P \leq 0.05$).

Similarly, in *T. pratense* the concentrations of the JA-Ile precursors: OPDA and JA were also consistently lower over time after the infestation with the native TR clone than levels determined after infestation with non-native clones MR and PR (Figure 5A and B). Moreover, at 48 hours after infestation with the native aphid TR clone, OPDA reach levels even lower than constitutive levels found in uninfested control plants (Figure 5A, Table S2). This strong modulation was also visible in the JA structurally related derivative: OH-JA2 (Figure 5E), whereas the other JA derivative, OH-JA1, was not detectable in *T. pratense*. In the same direction of his precursor (JA-Ile), the concentrations of the hydroxylated derivative HO-JA-Ile were always higher in plants infested with the non-native MR and PR clones than after the infestation with the native TR clone (Figures 5F). On the other hand, levels of the carboxylated JA-Ile derivative fluctuated without evidence of a specific pattern, but still keeping the levels in plants infested with the native TR clone as the most similar to levels found in uninfested control plants (Figure 5G).

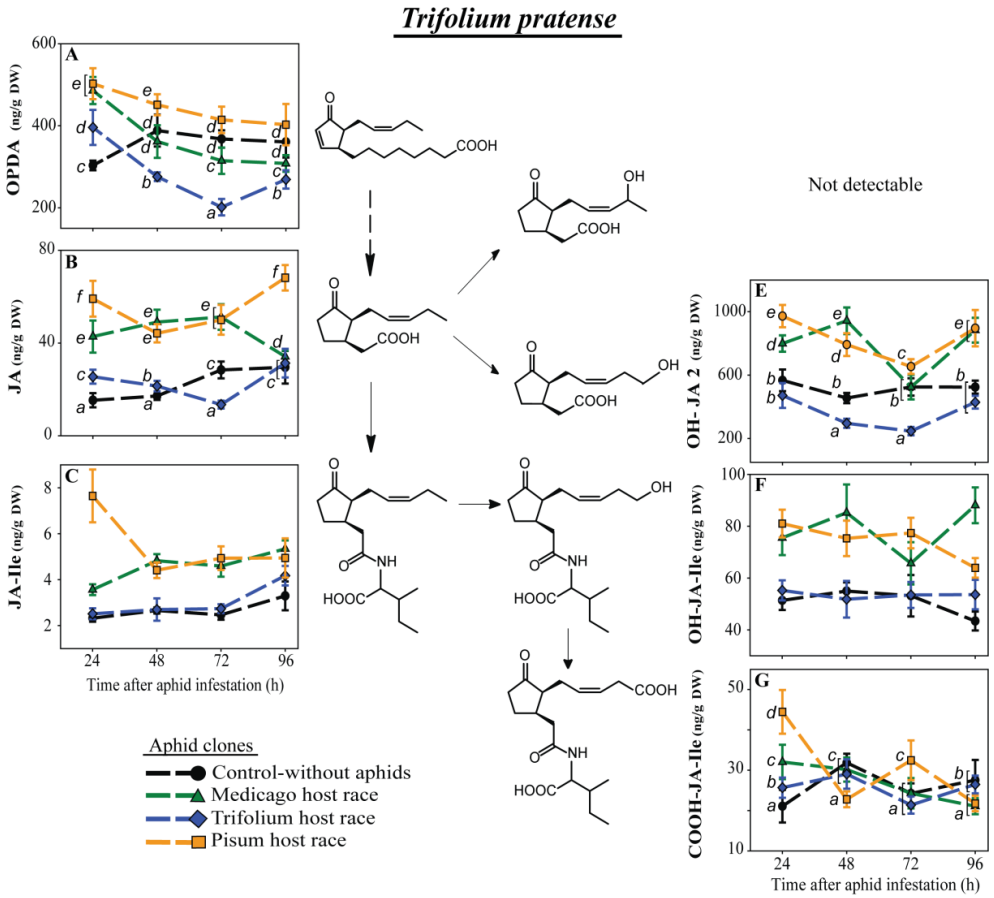

Figure 5. Level of JA pathway metabolites in *T. pratense* plants after infestation with pea aphid clones of different host races. Symbols represent means ± SE. Statistical values are presented in Table S4. In cases where a significant influence of the aphid clone on the phytohormone level was dependent on the time after aphid infestation (interaction), significant differences ($P \leq 0.05$) between aphid clones at different time points are indicated by different letters.

Unlike to the other plant species, in *P. sativum* the JA and JA-Ile structurally related derivatives were not detectable by the analytical method employed in our experiment (Figure 6). While both JA-Ile precursors, OPDA and JA were detected with concentrations changing over time in a clone dependent manner (Figure 6A and B, Table S2). The levels of these precursors after the infestation with the native PR clone were usually equivalent to levels determined in uninfested control plants.

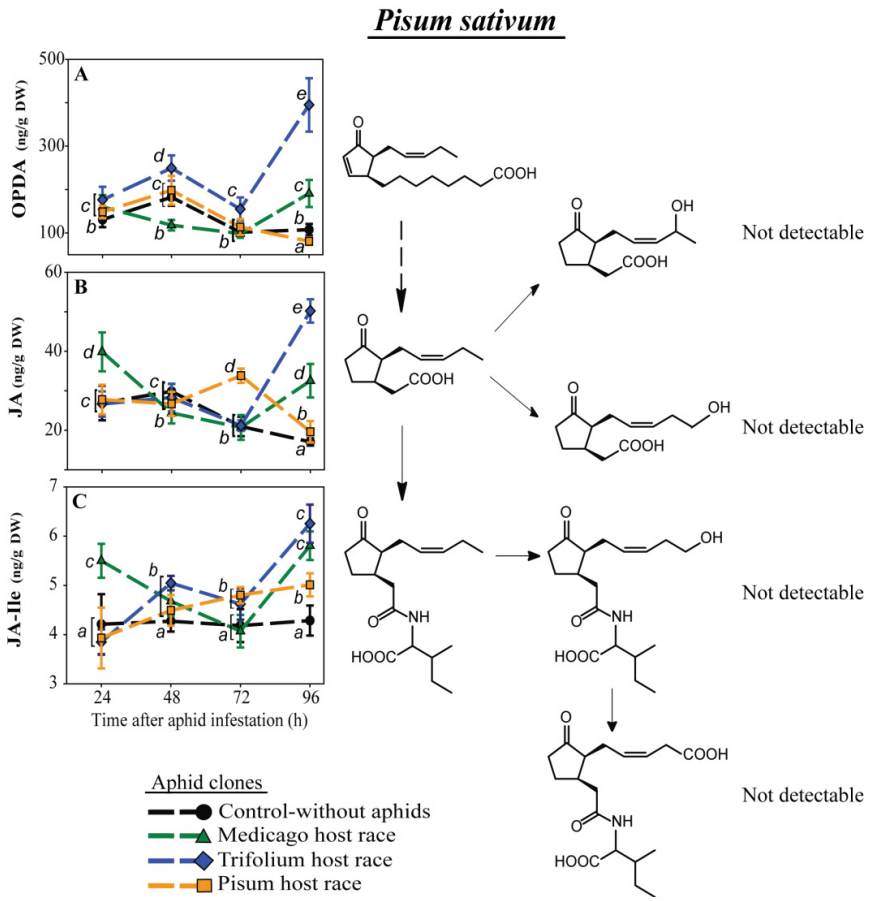

Figure 6. Level of JA pathway metabolites in *P. sativum* plants after infestation with pea aphid clones of different host races. Symbols represent means ± SE. Statistical values are presented in Table S4. Different letters indicate significant differences between treatments ($P \leq 0.05$).

Finally, in the universal host plant *V. faba*, the levels determined for the JA-Ile precursors, OPDA and JA, changed over time in an aphid-clone specific manner. The concentrations of these precursors were mostly higher in aphid-infested plants than in uninfested control plants, a tendency that was more marked in OPDA than JA (Figure 7A and B). After 96 hours of the aphid PR and TR clones infestations, OPDA levels were significantly lower than both MR clone infestation and uninfested control plants. JA concentrations at the same time point were equivalent to (for PR clone infestation) or lower than (for TR clone Infestation) those found in uninfested control plants (Figure 7A and B). In contrast, MR clone infestation elicited after 96 hours a high increase of JA levels (Figure 7B). This increment in the concentrations at 96 hours was also determined for all JA structurally related derivatives (JA-Ile, OH-JA1, OH-JA2, and 12-OH-JA-Ile) after MR clone infestation (Figure 7B-F). After infestation with TR and PR clones, the levels of the JA derivative, OH-JA1, decreased over time to even lower levels than uninfested control plants (Figure 7D). On the other hand, the

levels of OH-JA2 and 12-OH-JA-Ile in *V. faba* plants infested with the same clones were equivalent to those found in uninfested control plants over time (Figure 7E and F). The carboxylated form of JA-Ile, 12-COOH-JA-Ile, could not be detected with our method in *V. faba*.

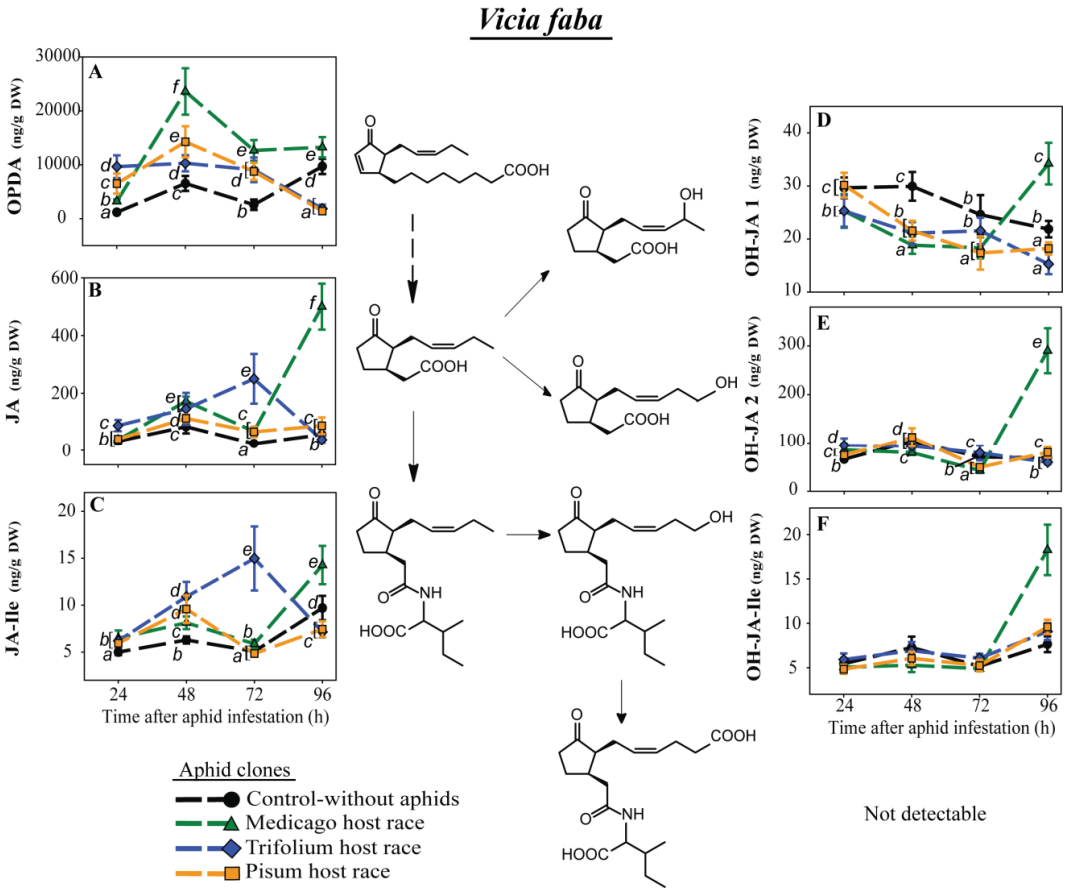

Figure 7. Level of JA pathway metabolites in *V. faba* plants after infestation with pea aphid clones of different host races. Symbols represent means ± SE. Statistical values are presented in Table S4. In cases where a significant influence of the aphid clone on the phytohormone level was dependent on the time after aphid infestation (interaction), significant differences ($P \leq 0.05$) between aphid clones at different time points are indicated by different letters.

There were minor changes over time in the levels of the JA-Ile precursors and derivatives of uninfested control plants, that may be attributed to plant developmental changes or attempts to mimic on these uninfested plants the experimental manipulations performed during the infestation of the plants (enclosure in an air-permeable cellophane bag to prevent aphid escape, leaf brushing to remove aphids before sampling the plant material)

2.4 Discussion

2.4.1 Infestation with native pea aphid host races leads to lower jasmonate and salicylate signaling

The phytohormone response patterns observed during our experiments provide new evidence about the capacity of native pea aphid host races to modulate plant defenses. When legume plants were infested with clones of different pea aphid host races, several distinct patterns of phytohormone levels were obtained depending on the legume species, the pea aphid clone, plant-aphid compatibility, and the duration of the aphid infestation. In *M. sativa* and *T. pratense*, the concentrations of the JA active form, JA-Ile, strongly correlated with the observed aphid clones performance. In both plants, non-native aphid clones infestations trigger the synthesis of high levels of JA-Ile, whereas native aphid clones infestations led to a much lower induction of the synthesis(MR on *M. sativa*) or even to a near complete lack of elicitation (TR on *T. pratense*). The moderated induction of this phytohormone could indicate: 1) a complete absence of the aphid recognition by the plant or 2) an active modulation of the biosynthesis by the native aphid clones. The second option seems to be more reasonable considering that the JA signaling responses are usually activated upon insect wounding or damage. As phloem feeders, aphids have to penetrate first the plant tissues, and in this path for reaching the phloem sap they regularly pierce and salivate into cells. Since aphids spend more time in this penetration phase when they are feeding on native than on non-native host plants (Schwarzkopf et al., 2013), possibly involving a higher number of cell punctures, they can cause more tissue damage on their native host. An outcome of this tissue damage should be a stronger activation of the JA pathway by the native aphid clones than by non-native clones. However, this was not the case in our experiments, suggesting that pea aphids feeding on their native host plants were either able to hide the tissue damage they caused from plant recognition systems or to suppress the defense response capacity of the host plants by modulating the biosynthesis of JA-Ile. The plant defense suppression hypothesis is supported by findings in the literature showing how the previous infestation with pea aphids resulted in increased performance of conspecific offspring (Takemoto et al., 2013). Similarly, for other aphid species like the soybean aphid (*Aphis glycines)* has been found that can increase the suitability of host plants for their conspecifics by inducing susceptibility (Varenhorst et al., 2015). Our assumption also infers that a substantial

increase in the JA defense signaling could be responsible for the observed low performance of the non-native aphid clones.

Several lines of evidence confirm the efficacy of the JA signaling against these phloem feeders in other plant-aphid interactions. First, Ellis et al. (2002) found that in *A. thaliana* the activation of JA signal pathway enhanced resistance against *Myzus persicae*. Second, genomic studies showed that after infestation with the blue-green aphid (*Acyrthosiphon kondoi*), 10 of 13 genes associated with the JA pathway were induced exclusively in resistant *Medicago truncatula* plants, but not in the susceptible ones, whereas all SA-responsive genes tested were induced in both resistance and susceptible plants (Gao et al., 2007).

Although we found a general negative association between aphid performance and JA-Ile levels over time, a similar association for SA was only partially detected. The lack of complete negative association was observed after 96 hours in *M. sativa* plants, where the native MR clone elicited the synthesis of SA and JA-Ile levels to equal or higher concentrations than those elicited by the infestation with the non-native aphid clones. Nevertheless, the native MR clone survived and developed on its native host much better than non-native clones indicating its ability to cope with both the constitutive and any induced defense of the plant (Walling, 2008).The increase in SA levels and up-regulation of SA-related genes after aphid infestations have been previously reported in the literature (Moran and Thompson, 2001;De Vos and Jander, 2009;Mai et al., 2014;Zhang et al., 2015;Stewart et al., 2016), reason why we should not ignore the relevance of this phytohormone in our system. Such increased in SA plant related signaling might be caused not only due to the incremented number of MR aphids, which could influence the level of defense responses (Mai et al., 2014;Stewart et al., 2016) but also by the induction generated by factors from aphid endosymbionts which might enter the plants cells via aphid saliva. For instance, the GroEL protein of the obligate aphid endosymbiont *Buchnera aphidicola*, induced the SA-defense marker gene expression, and transgenic *A. thaliana* lines expressing GroEL exhibited a significant but small reduction in aphid fecundity (Chaudhary et al., 2014). Thus SA related defense triggered by endosymbionts led to a fitness cost but not high enough to prevent aphid increase. Regardless of whether the JA or SA defense pathway was most effective against non-native aphids in our experiments, our measurements of aphid performance and phytohormone levels suggest that the native aphid clones (clone TR on *T.*

pratense, clone MR on *M. sativa*) were able to modulate *M. sativa* and *T. pratense* defenses on for their benefit.

Different from *M. sativa* and *T. pratense* phytohormone patterns, in *P. sativum* the infestation with the non-native MR and TR clones only trigger minor but fluctuating plant responses over time, reflecting their substantial survival, growth, and reproduction measured in our experiments and in previous studies (Schwarzkopf et al., 2013). The only exception was observed at 96 hours after infestation when the non-native clones elicited the synthesis of higher JA-Ile levels than the native PR clone. Similar fluctuations to the ones observed for SA and JA-Ile levels have also been reported for JA and JA methyl ester in *P. sativum* plants after pea aphid infestation (Mai et al., 2014). JA-related gene transcripts also showed fluctuations in *A. thaliana* after infestations with *Brevicoryne brassicae* (Kusnierczyk et al., 2008). Assuming that these fluctuations in the phytohormone levels were an expression of the intermediate ability of the non-native aphids to deal with the plant response remains an open question. Aphid performance may be a consequence of their influence on plant defense signaling pathways or their tolerance of defense toxins, deterrents, and phloem-sealing mechanisms.

On the 'universal host', *V. faba*, both JA, and SA regulated plant defenses are ineffective against the pea aphid since clones of all host races performed very well, even better than in the other host plants. The positive modulation of *V. faba* defenses by pea aphis for their benefit has been reported by Takemoto et al. (2013), they observed a faster development of *A. pisum* nymphs when they feed in previously infested *V. faba* plants with the same pea aphids. Since these pre-infested plants showed lower JA levels than uninfested control plants, a modulation of the JA-related defenses was assumed. The pattern of phytohormone changes in this species was different from those observed in the other host plants. SA levels after the infestation with all clones were kept low during most of the experiment. However, after 96 hours the levels were rose significantly concerning those measured in uninfested control plants, without affecting the performance of the aphids, what confirmed that SA signaling is not contributing to an active defense against pea aphids in *V. faba*. In contrast, JA- Ile levels generally rose over the whole experiment, but interestingly JA-Ile levels after 96 hours of infestations with the TR and PR aphid clones were even lower than those of uninfested control plants, negatively correlating with their higher performance at the same time point.

ABA, a phytohormone long known for participating in plant growth regulation (Cutler et al., 2010), protection under water stress conditions (Schroeder et al., 2001), control seed dormancy and germination (Karssen et al., 1983), and source-sink communication (Yu et al., 2015), has been recently included in the list of molecules involved in the modulation of plant defenses (Mauch-Mani and Mauch, 2005;Ton et al., 2009;Pieterse et al., 2012). Some reports showed evidence of the interaction of ABA with the JA and SA defense pathways. For example, upon wounding or herbivore damage, ABA can act synergistically with the MYC branch of the JA pathway leading to an increased resistance to herbivory (Anderson et al., 2004;Yasuda et al., 2008). On the other hand, ABA can also be able to suppress SA-dependent defenses (De Torres Zabala et al., 2009;Jiang et al., 2010;Cao et al., 2011). Previous studies involving aphids showed that infestations with these insects could generate inductions in the expression of ABA-regulated genes or an increase in the ABA levels in plants like *Glycine max*, *M. truncatula,* and *A. thaliana* (Studham and MacIntosh, 2013;Guo et al., 2015;Sun et al., 2015;Hillwig et al., 2016). In contrast, In our study, ABA levels in aphid-infested plants were generally lower or equivalent than those measured in uninfested control plants. A similar pattern was found by Stewart et al. (2016) in *M. truncatula plants* infested with *A. pisum*. Since ABA concentration profiles are neither plant- or aphid-dependent, we believe that ABA does not have an essential role in the modulation of the defense responses against pea aphids in legumes. However, we should not ignore that ABA could play other vital roles in plant-aphid interactions. For example, the role of ABA controlling the stomata closure under drought stress, and maintaining the plant turgor that could be beneficial for aphids during their feeding process (Guo et al., 2015). However, the stomata closure generates substantial reductions in the photosynthetic activity, what could decrease the availability of carbohydrates for aphid ingestion.

2.4.2 Native pea aphid host races may modulate jasmonate signaling by controlling precursors biosynthesis or changing the fate of JA and JA-Ile

To explore the mechanism by which native aphid clones might modulate the biosynthesis of JA-Ile, we continued using a targeted metabolomic approach to measure the levels of JA-Ile precursors and derivatives after the infestation with clones of various host races. It is reasonable to think that the lower JA-Ile levels observed after native clones infestations might be product of either a reduced biosynthesis of the precursors OPDA and JA, or an

increased biosynthesis of the hydroxylated and carboxylated JA and JA-Ile forms (OH-JA1, OH-JA2, OH-JA-Ile, COOH-JA-Ile), which could inactivate JA signaling (Miersch et al., 2008;Koo and Howe, 2012;Koo et al., 2014).

The levels of OPDA, first metabolite of the JA Pathway that we measured, in *M. sativa* and *T. pratense* plants were always significantly lower after the infestations with the native aphid clones (MR for *M. sativa* and TR for *T. pratense*) than after non-native clones infestations, correlating with the observed trends in the JA-Ile concentrations. In contrast, OPDA levels in the 'universal host' plant, *V. faba,* were incremented all along the first 72 hours after aphid clones infestations, but after 96 hours of infestations with the TR and PT clones, the biosynthesis of OPDA was reduced to levels below levels found in uninfested control plants. These findings suggest that aphids could be modulating the JA pathway either by reducing the availability of the OPDA precursors or by regulating the expression of the genes encoding the enzymes involved in OPDA biosynthesis. First, the reduction of OPDA precursors could begin with the control of the synthesis of α-linolenic acid (18:3), the first fatty acid substrate of the JA pathway produced from galactolipids of chloroplast membranes (Wasternack and Hause, 2013). Recently Kanobe et al. (2015) found that soybean plants (*Glycine max*) infested with the soybean aphid (*Aphis glycine*) present lower levels of α-linolenic acid than those ones measured after infestation with other type of antagonist like the soybean cyst nematode (*Heterodera glycines*) or the brown stem rot (*Cadophora gregata*). These findings suggest that specific pea aphid clones could have the ability to either reduce the biosynthesis of α-linolenic acid or to suppress the galactolipids hydrolysis. Second, OPDA is synthesized from the α-Linolenic acid by the sequential action of three enzymes: a lipoxygenase (LOX), allene oxide cyclase (AOC) and allene oxide synthase (AOS) (Wasternack and Hause, 2013), and their expression could be regulated. A previous study showed that the activity of LOX increased upon aphid infestation (Mai et al., 2014). But, has also been reported that the expressions of the genes encoding LOX and AOS were upregulated more strongly in wheat after infestations with an incompatible (non-native) biotype of the Russian wheat aphid (*Diuraphis noxia*) than after infestations with a compatible biotype (Liu et al., 2011). These studies indicate that compatible (native) pea aphid clones might be able to modulate the biosynthesis of OPDA by downregulating the expressions of *LOX* and *AOS* genes.

Pea aphids might also be able to modulate the JA-Ile levels by promoting the biosynthesis of the derivatives JA and JA-Ile hydroxylated and carboxylated forms. These JA and JA- Ile structurally related derivatives might play an essential role in the partial switch-off of JA signaling (Miersch et al., 2008). In our experiments, the abundance patterns of JA and JA-Ile derivatives were mostly correlated with those of their precursors JA and JA-Ile, and often higher in plants infested with non-adapted than adapted clones. Such equivalence suggests that the biosynthesis of these derivatives might not be the central strategy that native aphid clones have to modulate the JA signaling. However, all the increments in JA and JA-Ile concentrations in *M. sativa* and *V. faba* plants after 96 hours of infestations with the MR aphid clone corresponded with analogous raises in the concentrations of the JA and JA-Ile derivatives at the same time points. Thus native aphid clones might promote the biosynthesis of JA and JA-Ile derivatives as an alternative strategy to cope with high constitutive or increased levels of JA and JA-Ile molecules (Walling, 2008).

3 Research Chapter 2 . Non-targeted metabolomic approach reveals aphid race-specific changes in legume chemistry following infestation by pea aphids

3.1 Introduction

Since plant chemicals often serve as host finding and recognition cues (Storeck et al., 2000;Thöming and Norli, 2015), can also alter rates of food intake and food-utilization efficiency (Gabrys and Tjallingii, 2002;Kim et al., 2008), and can even influence survival of insects including aphids (Del Campo et al., 2003;Eleftherianos et al., 2006;Behmer et al., 2011), they might play a decisive role in host plant selection. Aphids do not only ingest plant chemicals together with the sugar-rich plant phloem sap, but also encounter them while navigating with their stylets through the intercellular spaces to reach the phloem cells. During this stage aphids frequently pierce epidermal and mesophyll cells, salivate into these cells and ingest some of their content (Pollard, 1973;Hewer et al., 2011;Moreno et al., 2011). The chemical information that aphids perceive during this process might be used to discard the plant or to continue with the plant tissue penetration and probably feeding process. The presence of attractive or stimulating secondary metabolites could be an important factor for the acceptance and colonization of a plant, whereas repellent metabolites would lead to a rejection of a plant (Powell et al., 2006;Pompon and Pelletier, 2012;Schwarzkopf et al., 2013).

The role of several types of secondary metabolites in the plant selection process by the pea aphid has been identified through different experimental strategies. For example, lines of *M. sativa* differing in levels of saponins and phenolic compounds have been used to investigate pea aphid performance and feeding behavior. These studies showed a reduction of aphid populations growth on plant lines with higher levels of these metabolites (Goławska et al., 2006;Goławska and Łukasik, 2009). Also, selected saponins from *Quillaja saponaria* increased pea aphid mortality when added to the artificial diet (De Geyter et al., 2012). Diverse flavonoid glycoside forms from *M. sativa* were proven as biologically active over pea aphids, reducing their daily fecundity and xylem ingestion (Golawska and Lukasik, 2012;Goławska et al., 2012a). Also, nitrogen-containing compounds showed biological activity. Lupine species and

varieties which contained certain alkaloids were not accepted by the pea aphid (Kordan et al., 2012). Although targeted approaches revealed that some secondary metabolites and chemical groups affect pea aphid performance, in most of these studies just one plant species was used and often the pea aphid host race was unknown. Thus, the contribution of plant chemicals to the maintenance and performance of pea aphid host races on legume plants is still largely unknown.

Metabolomics encompasses a group of analytical strategies allowing large-scale analyses of metabolites in samples (e.g., organisms, cells, fluids, soil samples) at a specific moment (Fiehn, 2002;Nakabayashi and Saito, 2013). The progress of analytical techniques allows a high throughput analysis of plant metabolites, facilitating approaches like metabolic fingerprinting and profiling (Fiehn, 2002). Whereas in the metabolic profiling a selected number of pre-defined metabolites is identified and quantified, the aim of the metabolic fingerprinting is the comparison of complete metabolomes of different samples without previous knowledge of their chemistry (Krastanov, 2010;Maier et al., 2010). Recently, Macel et al. (2010) introduced the term 'Ecological metabolomics' referring to the use of metabolomics in an ecological context. Several studies using these non-targeted eco-metabolomic methods demonstrated their effectivity for identification of secondary metabolites involved in plant-insect interactions (Arany et al., 2008;Marti et al., 2013;Sato et al., 2013), as well as in the study of its ecological implications (Riipi et al., 2004;Sardans et al., 2011;Richards et al., 2015). However, up to now, just a few studies applied metabolomic approaches to identify metabolites involved in plant-aphid interactions (Plischke et al., 2012;Hodge et al., 2013;Sato et al., 2013;Tzin et al., 2015). Recently, Hopkins et al. (2017) combined a metabolomic profiling approach with behavioral tests to understand the chemical signatures that underlie host preferences by *A. pisum*, but his study was limited to *Medicago* and *Trifolium* genus with two *A. pisum* host races.

To find metabolites potentially involved in the maintenance of pea aphid host races we applied a non-targeted mass spectrometry-based metabolomic approach. Polar and semi-polar fractions of three native host plants of the pea aphid, *M. sativa*, *P. sativum*, *T. pratense*, and the universal host *V. faba*, each infested with one native and two non-native aphid clones, as well as fractions of uninfested control plants were investigated.

3.2 Materials and Methods

3.2.1 Plant material

Four legume plant species, *Medicago sativa* cultivar (cv). 'Giulia' (alfalfa), *Trifolium pratense* cv. 'Dajana' (red clover), *Pisum sativum* cv. 'Baccara' (pea), and *Vicia faba* cv. 'The Sutton' (broad bean), were grown in 10-cm diameter plastic pots with a standardized soil mixture (7:20 mixture of Klasmann Tonsubstrat and Klasmann Kultursubstrat TS1, Klasmann-Deilmann GmbH, Geeste, Germany), in climate chambers at 20 °C, 70 ± 10% relative humidity, and under 16 h light/8 h dark photoperiod. The plants were watered twice a week. To have a sufficient amount of plant material for the extraction of metabolites, *M. sativa* and *T. pratense* plants were used four weeks after sowing, while *P. sativum* and *V. faba* were used three weeks after sowing.

3.2.2 Aphids

Three pea aphid (*Acyrthosiphon pisum* Harris) clones, each representing one pea aphid host race, were used in the experiments: the clone L84 representing the Medicago race (here called MR), the clone T3-8V1 representing the Trifolium race (TR), and the clone Colmar representing the Pisum race (PR). Aphids were initially collected from their native host plants *T. pratense*, *M. sativa*, and *P. sativum*, respectively, and genotypically assigned to their respective host race (for detailed information see Table S1 in Peccoud (2009)). All aphids were reared on 4-week-old broad bean plants. To synchronize the age of the aphids for the experiments, five apterous female adults were placed on a broad bean plant and were allowed to reproduce for 48 h and were then removed from the plants. Nymphs were kept on the plants for nine days until they reached adulthood. Then they were transferred to new plants were they reproduced. This procedure was repeated until enough synchronized young adult aphids were available for the experiment. To avoid escape of aphids, all aphid containing plants were covered with air permeable cellophane bags (18.8 x 39 cm, Armin Zeller, Nachf. Schütz & Co, Langenthal, Switzerland), and placed in a climate chamber under the same conditions described for the plant material.

3.2.3 Experimental design

Five adult apterous female aphids of each host race were placed in magnetic clip-cages (Ø 3.5 cm), on leaves of each plant species (two leaves for *M. sativa* and *T. pratense*, one leaf for *P. sativum* and *V. faba* plants). Leaves from all four plant species enclosed in magnetic clip cages but without aphids served as controls. Ten replicates of each combination were employed. All the infested and control plants were placed in climate chambers at 20 °C, 70 ± 10% relative humidity, and under 16 h light/8 h dark photoperiod, for 48 hours.

3.2.4 Plant material sampling and metabolite extraction

For plant material sampling, the clip cages were carefully opened, and aphids were removed using a paintbrush. Control plants without aphids were brushed in the same way as aphid-infested plants, to control for possible induction of metabolomics changes due to contact with the paintbrush. Leaves enclosed in the clip cages were harvested and rapidly frozen in liquid nitrogen. Frozen samples were stored overnight in Eppendorf tubes (2 mL) at -80 °C and then freeze-dried for 48 h. Dried plant material was homogenized into a fine powder by adding three stainless steel beads (3 mm Ø) in each tube and vigorously shaking for 4 min in a paint shaker (Skandex shaker SO-10m, Fast & Fluid Management, Sassenheim, The Netherlands). Portions of 10 mg dried plant material were extracted with 1 ml ice-cold extraction solution containing 80% methanol acidified with 0.1% formic acid and 0.1µg/ml of L-(+)-α-phenylglycine (as lock mass internal standard). Samples were immediately vortexed for 10 s and continuously sonicated in a water bath at room temperature (20°C) for 15 min at a maximum frequency of 35 kHz. After centrifugation (10 min at 4,500 *g* and -10°C), supernatants were filtered using 0.45mm PTFE AcroPrep™ 96-well filtration plates (Pall Corporation, Port Washington, NY, USA) and a vacuum filtration unit. All filtered plant extracts were stored at -80°C until LC-Orbitrap-MS analysis.

3.2.5 Plant extract analysis

From each plant extract, 10 µl were analyzed using a UHPLC system of the Ultimate 3000 series RSLC (Dionex, Sunnyvale, CA, USA) connected to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). UHPLC was performed on an Acclaim™ C18 column (150×2.1 mm, 2.2 µm, Dionex) prefiltered by a C-18, 3.5µm guard

column (2.1x10mm, Waters, Dublin, Ireland). Separation was accomplished using a gradient of 0.1% (v/v) formic acid (eluent additive for LC-MS, Sigma-Aldrich, Steinheim, Germany) in water (solvent A) and 0.1% formic acid in acetonitrile (hyper grade for LC-MS, Merck, Darmstadt, Germany) (solvent B) as follows: 0 to 5 min isocratic 100% (v/v) A, 5 to 32 min gradient phase to 100% B, 32 to 42 min isocratic 100% B, 42 to 42.1 min gradient phase to 100% (v/v) A, 42.1 to 47 min isocratic 100% A. The flow rate was set to 300 µl min^{-1}. The electrospray ionization (ESI) source parameters were set to 4.5 kV spray voltage, 35 V capillary transfer voltage at a capillary temperature of 275 °C. The samples were measured in the negative (NI) and positive (PI) ionization modes in separate runs using 30,000 m/Δm resolving power (mass range of m/z 150–2000) in the Orbitrap mass analyzer. Xcalibur™ software (Thermo Fisher Scientific™, Waltham, MA, USA) was used for data acquisition and visualization.

3.2.6 Data preprocessing

Raw data files were converted to mzXML format files using an MSconvertGUI tool (ProteoWizard 3.0x software) (Chambers et al., 2012) and uploaded in the interactive XCMS online platform (Tautenhahn et al., 2012). Parameter settings for XCMS data processing were as follows: A multigroup analysis was run in a centWave mode for feature detection (Δ m/z = 2.5 ppm, minimum peak width =10 s, and maximum peak width = 60 s); the correction of the retention time was perform in a obiwarp method (profStep = 1); and for chromatogram alignment: minfrac = 0.5, bw = 5, mzwid = 0.015, max = 100, minsamp =1. Tables with the intensities of the detected features were obtained as output. Features which are missing in 3 or more out of 10 samples in all four treatments (four control plants, or one plant with four different treatments) were classified as sporadic features and were discarded from the dataset.

3.2.7 Data analysis

For the first colonization of plants by aphids, compounds which are characteristic of a certain plant species might play a crucial role. Most characteristic compounds are compounds which are unique to a given plant species. A feature was classified as unique to a certain plant species when it fulfilled the following criteria: It appeared in at least eight of ten samples of the

plant species of interest, and the feature was absent in the other plant species or appeared in not more than two out of ten samples per plant species.

To see whether and how the metabolic profiles differed between uninfested control plants, principal component analyses (PCAs) were performed with the web-based program MetaboAnalyst (Xia et al., 2009;Xia and Wishart, 2011). Due to technical reasons (limited number of features which can be processed in the program) 40 % of the features that were near-constant throughout the plant species based on the interquartile range were filtered out before analyses. Before the data were analyzed with a PCA features were mean-centered and divided by the standard deviation of each feature (is equivalent to auto-scaling) to make them comparable.

To see whether and how the metabolic profiles changed across pea aphid host race infestation within a given host plant, several PCAs were performed. Since all metabolic profiles in one comparison came from the same plant species, and only a fraction of the metabolome might change due to aphid infestation, it was assumed that many features might not differ between plants infested with different aphid host races and uninfested control plants. Therefore, PCA analyses were accomplished on the 5% of metabolomic features that changed most across biotype infestation within a given host plant. To identify the 5 % most differently regulated features, all features were compared by a non-parametric one-way ANOVA on ranks and sorted by their false discovery rate (FDR). Before PCA these 5 % most differently regulated features were normalized by log-transformation and scaled by mean-centering and division by the standard deviation of each feature (auto-scaling) to make them comparable.

To identify features that showed particular patterns of change, e.g., down-regulation in plants only when an adapted aphid race was feeding, but upregulation when non-adapted aphid races were trying to feed, the pattern hunter tool in the MetabolAnalyst software was used. Therefore, a Spearman rank correlation analyses was performed against given patterns. A pattern was specified as a series of numbers, were each number corresponded to the concentration levels of the features in the corresponding group. For instance, the pattern “2-1-2-2” corresponding to the groups “uninfested control plants – plants infested with the adapted aphid race – infested with non-adapted aphid race A – infested with non-adapted aphid race B” was searching for features down-regulated (positive correlation) or upregulated (negative

correlation) exclusively by the adapted aphid race. To test whether intensities of selected features differed among the four treatments, one -way analyses of variance (one-way ANOVA) were performed. In cases of significant differences, Tukey HSD tests were executed to reveal which groups were different from each other. These univariate analyses were conducted using R software version 3.2.0 (R Development Core Team, 2015).

Chemical groups were assigned to all the selected features through putative identifications by performing library mass, and spectrum matches in METLIN, Human Metabolome Database (HMDB), MetFrag, MassBank, and LipidMaps. No matches were considered as unknowns.

3.3 Results

3.3.1 Legume plants had different metabolic profiles

To evaluate how the metabolic profiles of the four uninfested plant species, *M. sativa, T. pratense, P. sativum* and *V. faba*, differed, principal component analyses (PCA) were conducted. PCA plots show a clear separation of all four plant species in both ionization modes. Biological replicates of each plant species were always grouped together with small confidence intervals. The first principal components (PC1) explained the 26.5 % and 24.8 % of the total variability for NI and PI datasets respectively, whereas the second principal components (PC2) accounted for the 20.8 % and 20.9 % (for NI and PI modes respectively) of the total variability of the data set (Figure 8A and B). For both ionization modes, the metabolic profiles of *M. sativa*, *T. pratense,* and *V. faba* were separated mainly along PC1. *P. sativum* metabolic profiles were separated from the ones of the other plant species along the PC2.

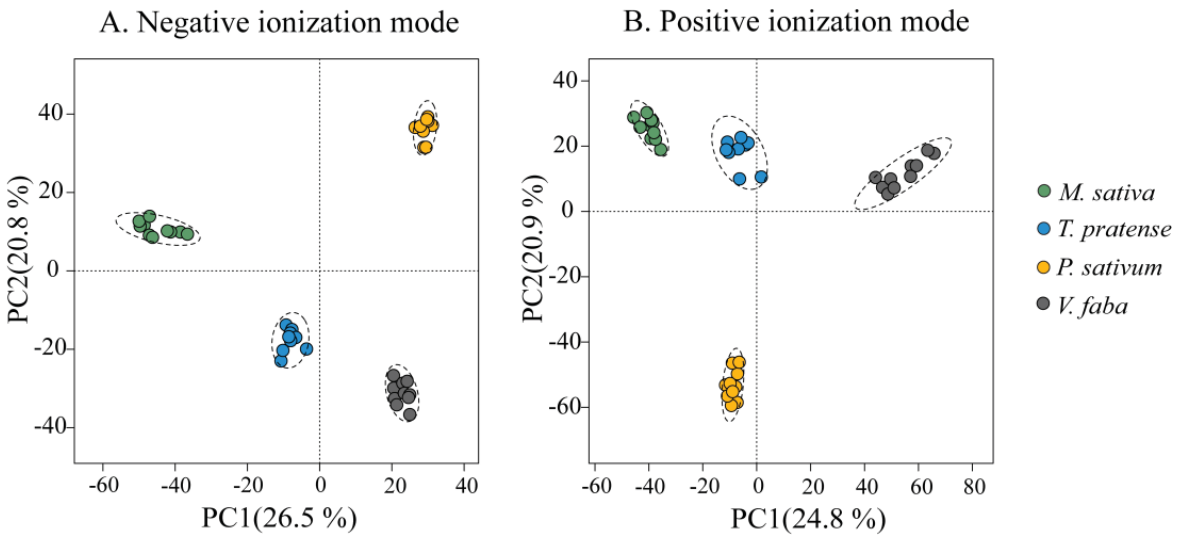

Figure 8. PCA plots based on metabolomic features from uninfested control plants in A) negative and B) positive ionization modes. Colored circles represent individual plant metabolic profiles. Dotted ellipses represent the 95% confidence regions for each group.

Most characteristic features of a plant species are those who are unique to a given plant species. To visualize the number of unique features as well as the magnitude of features shared between plant species, Venn diagrams were used (Figure 9). In both ionization modes, most features were shared between all four plants species (53.5 % and 72.6 % of all features for NI and PI respectively). Features unique to a certain plant species were much less common. Only 35.5 % (NI) and 19 % (PI) of all features were assigned to only one plant species. *M. sativa* and *T. pratense* plants possessed a higher number of unique features in comparison to *P. sativum* and *V. faba* plants, while *V. faba* displayed the lowest number of unique features. Additionally, *M. sativa* and *T. pratense* plants shared more common features, with 149 and 109 features for NI and PI modes respectively, than with *P. sativum* and *V. faba* plants (Figure 9A and B).

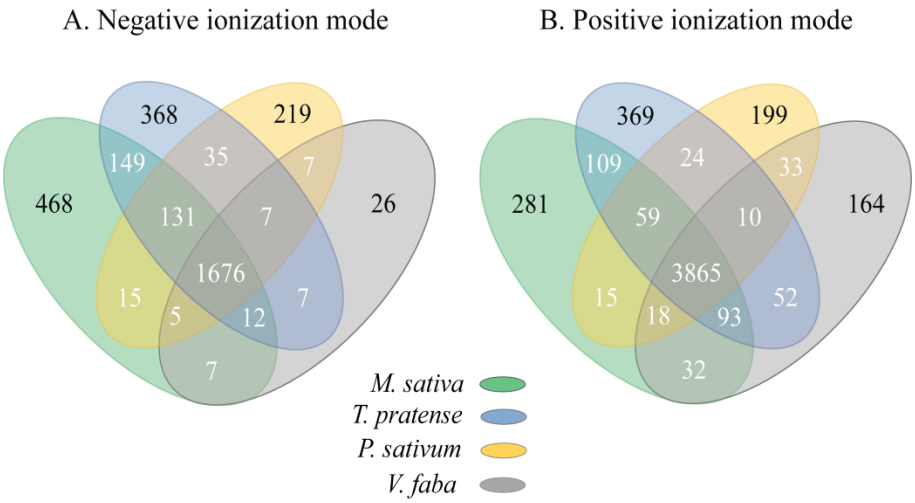

Figure 9. Venn diagrams based on metabolic features of uninfested control plants in A) negative and B) positive ionization modes. Features unique to a plant species are given in black, and common features among plant species are given in white. Colored areas represent each plant species.

For a pea aphid host race, metabolites unique to the host plant species could serve as host plant identification cues. Since these compounds could be responsible for the choice and acceptance of the host plant by the different pea aphid host races, we tentatively determined the number and identified the chemical groups of compounds of all unique compounds present in each plant species (Table 2).

As expected the number of unique plant compounds was reflected by the number of unique features. *M. sativa* was the plant species that displayed the highest number of unique metabolites with 107 and 86 in NI and PI mode respectively, followed by *T. pratense* with 103 and 83 metabolites, *P. sativum* with 57 (NI) and 53 (PI) metabolites, and *V. faba* with 13 (NI) and 67 (PI) metabolites. Flavonoid O-glycosides and peptides were the most common groups of compounds among all plant species. Among all the tentative identified metabolites, steroidal and triterpenic saponins were the most abundant and specific compounds in *M. sativa*. Additionally, glycosylated flavonoids, terpenes, and non-protein amino acids were also present in *M. sativa* among others. Most characteristic in *T. pratense* were isoflavonoids, flavanols, flavans, flavones, and anthocyanidins. *P. sativum* and *V. faba* did not show any specific compound classes. The unique compounds of both plant species belonged mainly to glycosylated flavonoids and peptides.

Table 1. The number of unique metabolites in uninfested *M. sativa*, *T. pratense*, *P. sativum* and *V. faba* plants and their tentative chemical classification.

Plant species	Negative ionization mode		Positive ionization mode	
	Number of unique metabolites	Tentative chemical groups	Number of unique metabolites	Tentative chemical groups
Medicago sativa	107	Benzoic acid esters Diacylglycerophosphoinositol biphosphates Disacharides Diterpenes Flavonoid *O*-glycosides Lignan glycosides Peptides Prostaglandins Steroidal glycosides Triterpene glycosides Unknowns	86	Flavonoid *O*-glycosides Isoprenoids α-aminoacids Non-protein aminoacids Peptides Triterpene glycosides Triterpens Unknowns
Trifolium pratense	103	Alpha amino acids Chalcones Coumaric acid esters Flavones Flavonoid *O*-glycosides Flavonols Glucoronic acid derivates Glycoaminoacid derivates Hydrolizable tannins *N*-acyl-α-amino acids Peptides Spirostanols Trihexoses Triterpene glycosides Unknowns	83	Flavans Flavonoid *O*-glycosides Isoflavonoids *N*-acyl-α-amino acids Peptides Phenolic glycosides Unknowns
Pisum sativum	57	*C*-Glycosyl compounds Flavans Flavonoid *O*-glycosides Peptides Unknowns	53	Dicarboxylic acids Flavonoid *O*-glycosides *N*-acyl-amines Peptides Unknowns
Vicia faba	13	Dihexoses Fatty acyls Flavonoid *O*-glycosides *O*-glycosyl compounds Peptides Saccharolipids Unknowns	67	Flavonoid *O*-glycosides Iridoid glycosides limonoids Peptides Unknowns

3.3.2 Legume metabolic profiles were modified differently by each pea aphid host race

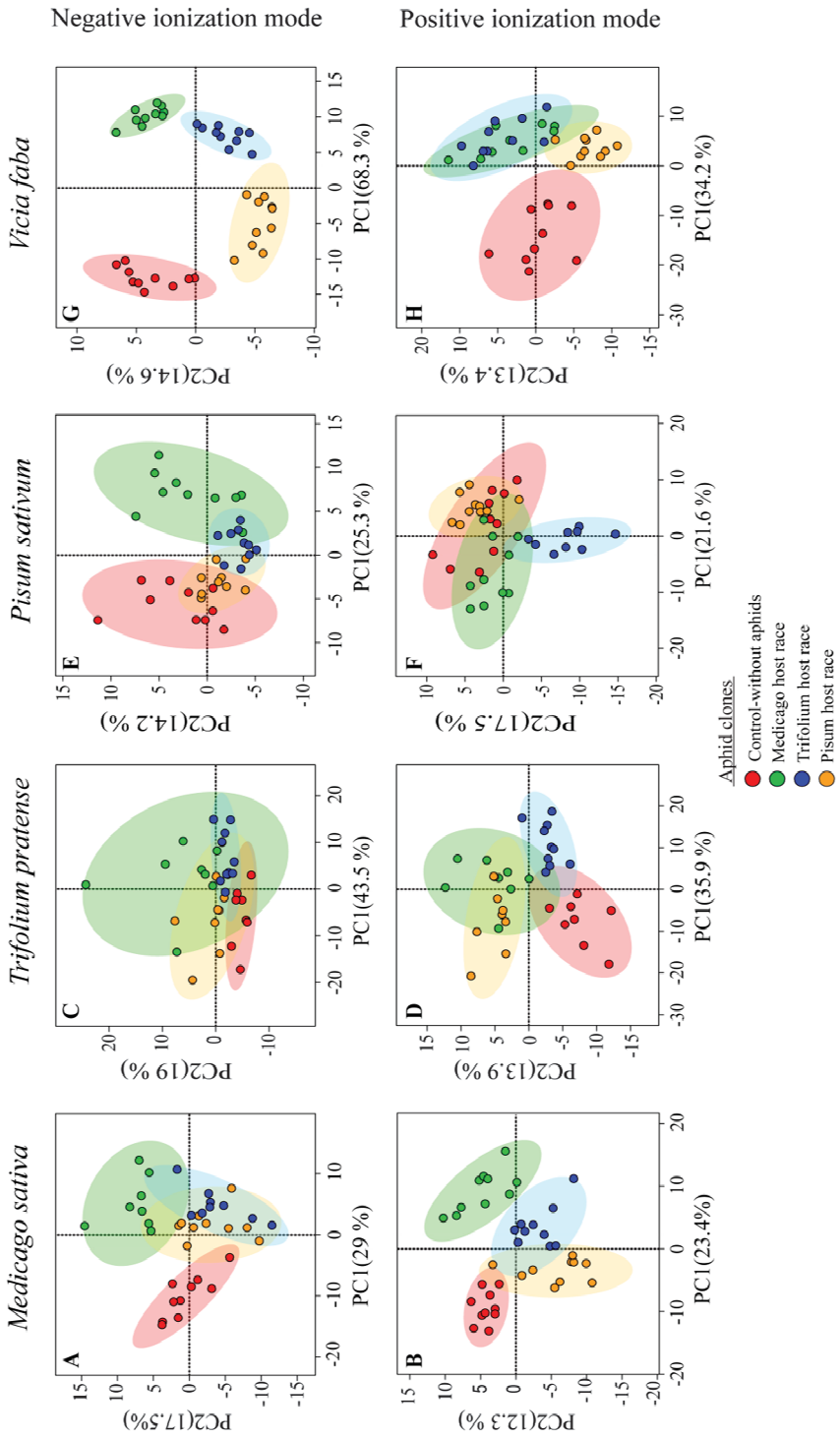

Figure 10. PCA plots based on the 5% of metabolomic features that changed most across host races infestation within a given host plant. Negative (on top) and positive (at the bottom) ionization modes. Small colored circles represent individual plant metabolic pro profiles after infestation with MR clone (green), TR clone (blue), PR clone (yellow) and of uninfested control plants (red). Colored ellipses represent the 95% confidence regions for each group.

To find out whether and how the plant metabolic profiles changed across the infestations with the various pea aphid host races, PCAs were performed based on the 5 % of metabolomic features that changed most across host races infestation. In general, metabolic profiles of uninfested control plants were separated from the metabolic profiles of aphid-infested plants. Thus the metabolic profiles of the plants changed upon aphid infestation. The degree of separation of metabolic profiles of plants infested with different pea aphid host races depended on the plant species. Whereas metabolic profiles of *V. faba* and *M. sativa* changed substantially depending on the attacked pea aphid host race, profiles of *T. pratense* and *P. sativum* changed to a smaller extent (Figure 10).

The first two principal components (PC1 and PC2) explained the 46.5 % (NI) and 35.7 % (PI) of the total variability in the metabolic profiles of *M. sativa* plants (Figure 10A and B). All metabolic profiles originated from aphid infested *M. sativa* plants were separated from profiles of uninfested control plants along the first axis. Additionally, the metabolic profiles of plants infested with the native MR clone were separated from those of plants infested with the non-native TR and PR clones along the second principal component, in both ionization modes (Figure 10A and B).

62.5 % (NI) and 49.8 % (PI) of the total variability in the metabolic profiles of *T. pratense* was explained by the first two principal components (Figure 10 C, D). Although in *T. pratense* the separation of the different metabolic profiles was not as evident as in *M. sativa*, all the metabolomic profiles of aphid-infested plants were also separated from those of uninfested control plants especially in the positive ionization mode (Figure 10D). Furthermore, metabolic profiles of plants infested with native TR clone were grouped apart from those of non-native MR and PR infestations, also especially in the positive ionization mode (Figure 10D).

The proportion of variability in the metabolic profiles of *P. sativum* which could be explained by the first two principal components was with 39.5 % (NI) and 39.1 % (PI) lower than in the other plant species. In contrast to *M. sativa*, *T. pratense* and *V. faba* where metabolic profiles of uninfested plants separated well from profiles of aphid-infested plants, in *P. sativum* the metabolic profiles of uninfested plants overlapped to some extent with those of plants infested with the native PR clone (Figure 10E, F). However, in the negative ionization mode, the metabolic profiles of plants infested with the non-native MR and TR clones were separated

from the ones of uninfested control plants along the first principal component (Figure 10E). In the positive ionization mode, the metabolic profiles of plants infested with non-native TR clone separated from the metabolic profiles of the other plants along the second principal component (Figure 10F).

A large proportion of the variability in metabolic profiles of the universal host plant *V. faba* could be explained by the first two principal component (82.9 % for NI and 47.6 % for PI). Especially metabolic profiles obtained by the negative ionization mode changed drastically across different host race infestations. There was a clear separation between infested and uninfested plants in both ionization modes along the first principal component (Figure 10G and H). The second principal component separated MR clone infested and uninfested plants from PR and TR clones infested plants based on metabolic profiles obtained in the negative ionization mode (Figure 10G).

3.3.3 Plant metabolites were specifically modulated by native aphid host races

Pea aphid host races clones performed much better on their native host plants than on other plants. Probably due to the presence of unique plant metabolites which might enhance the performance of native aphid host races but reduce the performance of non-native host races. Also, particular strategies to modulate the signaling molecules levels might help aphids to perform well on their native host plant (Research chapter 1). The changes in the levels of signaling molecules might be reflected in modulations of the whole metabolic profiles but also in the specific modulation of certain compounds. We specifically looked for metabolites which showed 1) significantly reduced levels exclusively after infestation with native aphid host races (down-regulation of potential detrimental compounds), and 2) significantly increased levels only after infestation with non-native aphid host races (induction of potential defense compounds) (Table 3).

Flavonoids were the most frequent class of compounds satisfying these criteria in all host plants (Table 3). *M. sativa* plants contained the highest number of modulated compounds, with 4 and 13 metabolites detected in the NI and PI modes respectively, followed by *T. pratense* with 4 (NI) and 10 (PI) metabolites, *P. sativum* contained just 2 and 3 compounds for NI and PI modes respectively, which were modulated in this specific way. In *M. sativa* a glycosylated triterpenic saponin was found, that satisfied the first modulation pattern. Additionally, jasmonic

acid derivatives, β-hydroxy acids, sterols, and tetrapyrroles were also part of the group of differentially modulated compounds. In *T. pratense* besides the flavonoids, non-protein amino acids, dialkyl ethers, and fatty alcohols showed specifically regulated patterns. In *P. sativum*, besides the detected flavonoid metabolite, non of the other detected metabolites could be tentatively identified.

Table 2. The number of compounds down-regulated exclusively after the infestation with native aphid host races or increased exclusively after the infestation with non-native aphid host races, and their tentative chemical classification identified for each plant species in negative and positive ionization modes.

Plant species	Negative ionization mode		Positive ionization mode	
	Number of metabolites	Tentative chemical groups	Number of metabolites	Tentative chemical groups
Medicago sativa	4	Chalcones Isoflavonoids Triterpene glycosides Unknowns	13	Anthocyanins Beta hydroxy acids Disaccharides Jasmonates Sterol lipids Tetrapyrroles Triterpene glycosides Unknowns
Trifolium pratense	4	Isoflavonoids Unknowns	10	Dialkyl ethers Fatty alcohols Non- protein aminoacid Unknowns
Pisum sativum	2	Unknowns	3	Flavonol *O*-glycosides Unknowns

Figure 11 exemplarily shows two of the specifically modulated compounds from *M. sativa*. The compound with m/z 1086.55, a glycosylated triterpenic saponin, was not only present in *M. sativa* plants but also exclusively down regulated when the plants were infested with the native MR clone (Figure 11A). On the other hand, the compound with m/z 695.30, tentatively classified as a glycosylated flavonoid, also occurred in *M. sativa* plants, but its levels were significantly increased by the infestation with non-native TR and PR clones but not after infestation with the native MR clone. After infestation with the native MR clone, the levels of this metabolite remained at similar levels compared to those of uninfested control plants (Figure 11B).

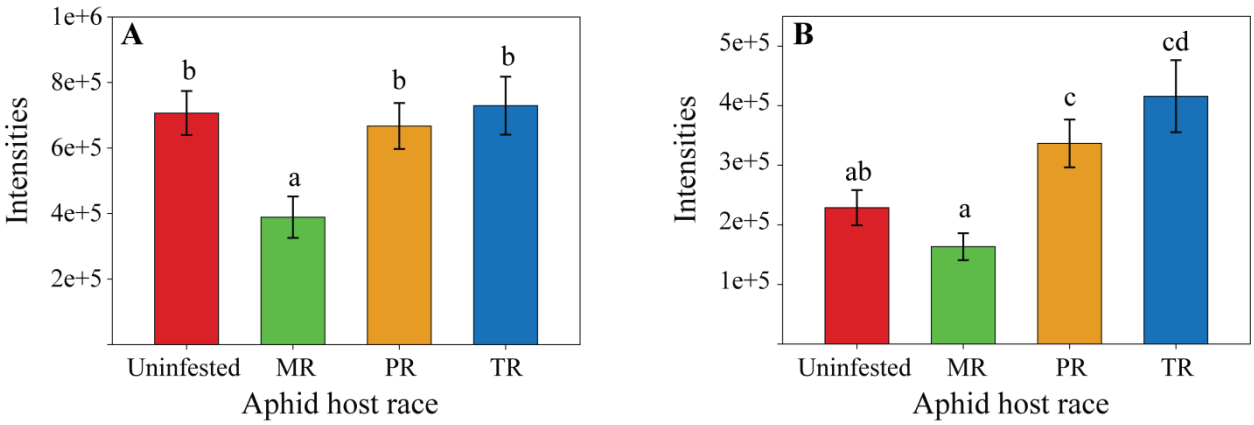

Figure 11. Metabolites from *M. sativa* with significantly reduced levels exclusively after infestation with native aphid host races (A) and with significantly increased levels after infestation with non-native aphid host races (B). A) Metabolite with m/z 1086.55, decreased by the native MR clone b) Metabolite with m/z 695.30 induced by the non-native PR and TR clones. Both metabolites were detected in negative ionization mode. Bars represent means ± se. MR–Medicago race, TR–Trifolium race, PR–Pisum race, Uninfested–uninfested control plants. Different letters indicate significant differences ($P \leq 0.05$) based on One-way ANOVA followed by Tukey HSD test.

3.4 Discussion

3.4.1 Legume host plants differed in their metabolic profiles

The separation of uninfested legume plants based on their metabolic profiles emphasized the existence of distinct chemical differences among them. Along the first principal component, *Medicago sativa and Trifolium pratense* were separated from *Pisum sativum* and *Vicia faba* (Figure 8A), while *P. sativum* and *V. faba* were well separated along the second principal component (Figure 8A and B). This separation indicates the existence of chemical differences between the four investigated plant species. This separation based on the metabolome was also supported by the existence of unique features in each plant species. It seems that *M. sativa* and *T. pratense* are chemically the more complex plant species since they contained a higher number of unique chemical features in comparison to *P. sativum* and *V. faba* (Figure 9). Additionally, *M. sativa* shared more common features with *T. pratense* than with the other plant species, what makes them more chemically related. These chemical similarities go along with the phylogenetic relationship of *M. sativa* and *T. pratense* which are sisters in the monophyletic Trifolieae tribe (Wojciechowski et al., 2004;The Legume Phylogeny Working Group, 2013;Smýkal et al., 2015). The chemical complexity of *M. sativa* and *T. pratense* might

influence the performance of pea aphid races, as it was observed in our experiments (Sanchez-Arcos et al., 2016) and in previous studies (Goļawska, 2010;Schwarzkopf et al., 2013). These two plants might have developed a more complex and powerful armor of chemicals to defend themselves from the attack of herbivores including phloem feeders, and only those aphid races who have evolved strategies to cope with those chemical defenses will perform better on these plants.

Separated from *M. sativa* and *T. pratense* along the first principal component (NI, Figure 8A) were *P. sativum* and *V. faba*. Both plants belong to the Vicieae tribe (also known as Fabeae) (Steele and Wojciechowski, 2003;Wojciechowski et al., 2004). The chemical complexity of both plants was lower than the one of *M. sativa* and *T. pratense*. They contain much less unique chemical features (Figure 9) which might be the reason for the intermediate performance of most of the pea aphid clones on *P. sativum* and good development of all aphid clones on the universal host plant *V. faba*. However, *P. sativum* plants also separated from *V. faba* plants (Figure 8A and B). This is also reflected in phylogenetic data. Although *P. sativum* and *V. faba* are sisters in the Vicieae tribe, they do not share a common evolutionary ancestor (Steele and Wojciechowski, 2003). *V. faba* remains as a particular case in the group of cultivated legumes because its wild ancestor is not known so far (Smýkal et al., 2015). *V. faba* was also the plant species with the lowest number of unique features in comparison to the other plant species, what probably makes it suitable for all pea aphid host races and therefore, to be the universal host plant.

The most abundant unique chemical compounds found in *M. sativa* were steroidal and triterpenic saponins. Saponins are distributed in many families in the plant kingdom, but they are more frequently found in legumes (Vincken et al., 2007). Within the Leguminosae family, the *Medicago* genus is well known for containing a complex mixture of glycosylated triterpene saponins with a broad spectrum of biological properties (Carelli et al., 2015). However, triterpene saponins have also been found in *Trifolium* species (Kolodziejczyk-Czepas, 2012), supporting our identification of some glycosylated triterpene saponins in *T. pratense* plants. Saponins are a group of bioactive chemical compounds. They can act as defense compounds against pathogens (Sparg et al., 2004) and insect herbivores (De Geyter et al., 2007;Chaieb, 2010). Various studies reported antifungal (Oleszek et al., 1990;Wyman-Simpson et al., 1991;Lee et al., 2001;Abbruscato et al., 2014), nematicidal (D'Addabbo et al., 2011) and

insecticidal (Applebaum et al., 1969;Tava and Odoardi, 1996;Nielsen et al., 2010;Augustin et al., 2012;Da Silva et al., 2012) activities of plant saponins. Furthermore, some exciting studies have demonstrated that saponins from plants act against aphids (Krzymanska et al., 1983;Soule et al., 2000;De Geyter et al., 2012;Goławska et al., 2012b;Goławska et al., 2014). These demonstrated detrimental activities of saponins against aphids, and the singularity of these compounds in *M. sativa* could be an important factor for the observed resistance of this plant to some pea aphid clones (Schwarzkopf et al., 2013;Sanchez-Arcos et al., 2016). Besides saponins, we found other metabolites from various chemical groups in *M. sativa* that have been previously reported in this plant species or in the *Medicago* genus. e.g., flavonoids (Farag et al., 2007;Silva et al., 2013), peptides (Van Sumere et al., 1980), non-protein amino acids (Wilding and Stahmann, 1962), and benzoic acid esters (Newby et al., 1980), among others.

Although flavonoid compounds were found in all plant species, this group of metabolites was the most abundant and diverse in *T. pratense* plants (Dewick, 1977;He et al., 1996;Sivakumaran et al., 2004;Polasek et al., 2007;Saviranta et al., 2010b). Flavonoids are widely distributed in all plant species (Iwashina, 2000), but legumes are considered as an excellent source of these compounds (Velázquez et al., 2010). Flavonoids are divided into various classes (Iwashina, 2000) like isoflavones, flavones, flavonols, flavans, and flavonoid *O*-glycosides of which isoflavones are the most described class in this plant species (Biggs and Lane, 1978;Vetter, 1995;De Rijke et al., 2001;Klejdus et al., 2001;de Rijke et al., 2004;Swinny and Ryan, 2005;Toebes et al., 2005;Tsao et al., 2006;Oleszek et al., 2007;Drenin et al., 2008;Saviranta et al., 2010a;Drenin et al., 2011). The antioxidant capacity of flavonoids was extensively studied mainly due to their role in the abiotic stress response (Reuber et al., 1996;Agati et al., 2012). Many other studies reported that flavonoids are involved in plant defense against pathogens (Schlösser, 1993;Grayer and Harborne, 1994;Rauha et al., 2000), and can be phagostimulant, deterrent or toxic for insects (Bernays et al., 1991;Brignolas et al., 1998;Hoffmann-Campo et al., 2001;Widstrom and Snook, 2001;Haribal and Feeny, 2003). The significant number and diversity of flavonoid compounds we found in *T. pratense* could help this plant to defend against aphids, given the existing evidence of the activity of these compounds against phloem feeders (Montgomery and Arn, 1974;Lattanzio et al., 2000;Ateyyat et al., 2012). Besides the negative impact on aphids, some flavonoids can also have positive effects on herbivores. Thus they can induce insect oviposition (Ohsugi et al., 1985) or to sustain

insect feeding at certain concentration levels (Bernays et al., 1991;Lin and Mullin, 1999). Flavonoids in *V. faba* might, therefore, belong to the group which has positive effects on insects and might stimulate the plant-aphid interaction.

Additionally to flavonoids, different peptides were tentatively identified in all plants species (Table 2). Plant peptides can have antimicrobial properties (Nawrot et al., 2014;Tam et al., 2015). Thus these plant peptides might not have a direct effect on aphids but might harm the obligate aphid endosymbiont *Buchnera aphidicola* (Wilkinson, 1998), who is indispensable for the survival of the aphids due to their continue supplement of essential amino acids ,and nutrients that are not available in the plant (Douglas, 1998;Machado-Assefh et al., 2015).

Our non-targeted approach allowed us to identify differences in the chemical complexity of the plant species we used in this project. *M. sativa* and *T. pratense* were the plant species with the higher diversity of unique chemical compounds compared to *P. sativum* and *V. faba*. This chemical diversity might be responsible for the differences in the plant suitabilities for pea aphid clones or host races. Among all the chemical groups we tentatively identified saponins and flavonoids, that were not only the most abundant ones, especially in *M. sativa* and *T. pratense* respectively but also the ones with the most compelling evidence of activity against aphids.

3.4.2 Legume metabolic profiles were modulated differently by various aphid host races.

The metabolic profiles of the plant species were modulated in an aphid clone dependent manner. Specific metabolites were exclusively down-regulated by the native host races or upregulated only by the on native host races.

Metabolic profiles of *M. sativa* and *T. pratense* plants after the infestations with their respective native aphid clones were consistently different not only from those of uninfested control plants but also differentiated from profiles after the infestations with non-native clones (Figure 10A-D). In contrast, when the native PR clone fed on *P. sativum* plants the metabolic profiles remained similar to metabolic profiles of uninfested control plants, and only the infestation with non-native clones (MR and TR) generated changes in the plant metabolic profiles. Thus native aphid clones in this study either maintained (PR) or specifically changed

the chemistries (MR and TR) of their respective host plants. These two strategies might both lead to a reduced deterrence or toxicity of the hosts (Walling, 2008). Perhaps one of the most striking observations was that all the metabolic profiles of the universal host plant *V. faba* after infection with the different pea aphid clones differed (Figure 10G). However, along with the first principal component, the metabolic profile after PR clone infection was more similar to the metabolic profile of uninfested control plants, whereas metabolic profiles of MR and TR clone infested plants were more different to uninfested control plants (Figure 10G). Together with findings of the previous study where SA and JA- Ile levels were reduced upon MR and TR clones feeding, while after PR clone feeding the levels were kept equal to levels found in uninfested control plants (Sanchez-Arcos et al., 2016) this might indicate that pea aphid clones could probably really have the two different feeding strategies to cope with plant defenses.

The different modifications of the metabolic profile of plants by the various aphid clones or host races might be achieved through the secretion of aphid saliva into the plant. Once aphids have landed on a plant, they pierce the plant to reach the phloem sap (Hewer et al., 2011). In doing so, they puncture different cells and secrete saliva into the plant (Martin et al., 1997;Powell, 2005). Aphid saliva contains effectors which affect plant responses (Hogenhout and Bos, 2011;Elzinga and Jander, 2013;van Bel and Will, 2016). Evidence of plant defense suppression by aphid saliva was first reported by Will et al. (2007). They showed that salivary proteins (effectors) prevent the occlusion of sieve-tube elements by interactions with calcium molecules. Effector molecules present in the aphid saliva might be different between aphid host races or clones, leading to different plant defense responses. Such differences in defense signaling as observed for the JA and SA pathways in a previous study (Sanchez-Arcos et al., 2016), might generate changes in the biosynthesis of secondary metabolites that prevent or allow settling of aphids on a plant.

Several secondary metabolites which were either significantly reduced exclusively after the infestation with native aphid host races (down-regulation of potential detrimental compounds), or which were significantly increased only after the infestation with the non-native aphid host races (induction of potential defense compounds) and which might, therefore, play a role in the different performance of the various pea aphid host races on the different plant species, were tentatively identified (Table 3). One of these compounds was a glycosylated triterpene saponin, unique in *M. sativa* and exclusively down-regulated by the MR aphid clone

(Figure 11A). Saponins have already been reported to play a role in general plant resistance against insects (Applebaum et al., 1969;Potter and Kimmerer, 1989;Kreuger and Potter, 1994;Fields et al., 2010;Nielsen et al., 2010), but also in *M. sativa* (Nozzolillo et al., 1997;Adel et al., 2000;Agrell et al., 2003), and against aphids in particular (Soule et al., 2000;Goławska et al., 2012b;Goławska et al., 2014). The adverse effects of saponins against the pea aphid observed in several studies (Golawska, 2007;Goławska and Łukasik, 2009;De Geyter et al., 2012;Goławska et al., 2014) support the idea that the native MR clone down regulated a detrimental plant metabolite in its native host plant and showed, therefore, a good performance whilst non-adapted aphid races were not able to suppress the harmful compound and suffered from repellent, deterrent or toxic effects (Sanchez-Arcos et al., 2016).

One of the compounds which were exclusively up-regulated in *M. sativa* after infestation with non-native aphid races was most likely a flavonoid. Increased levels of flavonoids in legumes after pea aphid infestation were already reported by Morkunas et al. (2016). Goławska and colleagues (2008;2012;2014) demonstrated the detrimental effects of flavonoids on the pea aphid. Thus this tentatively identified flavonoid is a good candidate to be tested for its effect on non-native pea aphid host races. In *M. sativa*, compounds from other chemical groups like chalcones, jasmonic acid derivatives, lipids, and tetrapyrroles were also modulated in similar ways like the saponin and the flavonoid mentioned before, thus belonging to the group of selected candidate metabolites which needs to be tested for their effect on the different pea aphid host races.

Our non-targeted metabolomic approach represented a reproducible way to select plant secondary metabolites potentially involved in the choice and acceptance of the legume hosts by the various pea aphid clones or host races. In contrast to previous metabolomic studies where plant secondary metabolites were not associated to host plant discrimination by the pea aphids (Hopkins et al., 2017), we assume that probably saponins and flavonoids might be associated with the discrimination and compatibility of the pea aphid host races with the different legume species used in this project. These compounds might play an important role as repellents or deterrents and co-occur with those compounds with stimulating roles, leading to the recognition, and acceptance or rejection of host plants by the pea aphid host races (Del Campo et al., 2003).

4 Research Chapter 3. Pea aphid host races differentially modulate volatile organic compounds responses in legume plants

4.1 Introduction

Plant volatiles might serve as important cues in the selection of host plants by pea aphids and influence subsequent growth, survival, and fecundity (Pickett et al., 2013). The blends of volatiles released are often very characteristic of individual plant species, cultivars, organs and environmental conditions (Gen-ichiro et al., 2016). These substances are well known as attracts of other legume insects for feeding and oviposition. For example, the pea moth, *Cydia nigricana*, an important pest on some legumes is attracted and guided to oviposit by volatile blends from the sweet pea *Lathyrus odoratus*, (Thoming et al., 2014;Thöming and Norli, 2015). Similarly, the pea weevil, *Bruchus pisorum* is also strongly attracted by volatiles emitted by its host *Pisum sativum* (Ceballos et al., 2015). Furthermore, the leafminer, *Liriomyza sativae* shows strong electroantennogram (EAG) responses to volatiles associated with its host plant (Zhao and Kang, 2002). Aphids also employ volatile emission to find their host plants. The black bean aphid, *Aphis fabae*, uses an identified group of volatiles as a cue to locate its host plant, *Vicia faba* (Webster et al., 2008). However, plant volatiles can also act as insect repellents by signaling the unsuitability of the plant. For example, the aphid *Myzus persicae* was repelled by a mix of volatiles from wild potato species, *Solanum berthaultii,* and the cultivated potato, *S. tuberosum* L., probably as a way to avoid plants with non-optimal nutrition (Avé et al., 1987). Similarly, the Silverleaf whitefly, *Bemisia tabaci,* and associated natural enemies were repelled by aphid-induced plant volatiles (Tan and Liu, 2014), possible because aphids are also phloem feeders that might compete with whiteflies. Therefore, pea aphids might also be able to use volatile cues to establish the suitability of their host plants.

Plant volatiles not only serve as olfactory cues for insect herbivore host location but also can influence the behavior of predators and parasitoids of herbivores. Several studies showed that herbivore enemies are attracted to the volatile blends emitted by plants infested with their prey or hosts. For example, *Aphidius ervi*, a well-known parasitic wasp of *A. pisum* aphids, is significantly more attracted to plant infested with pea aphids than to uninfested plants

(Pennacchio-Tremblay, 1993), and also more attracted to blends of aphid-induced plant volatiles than to individual compounds from the blends (Takemoto and Takabayashi, 2015). *Aphidius ervi* is even attracted to the volatile blends emitted by plants treated exogenously with liquid diets upon which *A. pisum* aphids have been feeding (Takemoto and Takabayashi, 2012). Thus the protein effectors presented in aphid saliva and excreted into the artificial diet might be eliciting the biosynthesis of an attractive volatile blend for this parasitoid. Plant volatiles induced by aphids may also play a role in plants by priming the defenses of neighboring plants so that they produce defenses faster or in greater amounts upon subsequent herbivore attack (Heil and Ton, 2008). Studies have shown that maize plants exposed to insect herbivore-induced plant volatiles increased their defenses against subsequent herbivore attack (Ton et al., 2007;Engelberth et al., 2013).

Among the major chemical classes of plant volatile compounds, green leaf volatiles (GLVs) are perhaps best known for their roles as signals for herbivores, herbivore enemies, and other plants or plant parts (Scala et al., 2013). GLVs are biosynthesized by the hydroperoxide lyase (HPL) branch of the oxylipin pathway through the peroxidation of polyunsaturated fatty acids (Taurino et al., 2013). Jasmonates are also produced by peroxidation of polyunsaturated fatty acids, but their formation follows the allene oxide synthase (AOS) branch of the oxylipin pathway. The enzymes HPL and AOS are both members of the CYP74 subfamily of the cytochrome P450 family of enzymes (Dudareva et al., 2013;ul Hassan et al., 2015;Ameye et al., 2017). The GLVs comprise a broad group of volatiles consisting mainly of C6 or C9 aldehydes, alcohols and their ester derivatives, with the C6 compounds showing more activity in ecological interactions than the C9 compounds(Matsui et al., 2012;ul Hassan et al., 2015;Kunishima et al., 2016). Thus pea aphids might also be expected to respond to GLVs (Hildebrand et al., 1993;van Giessen et al., 1994).

Plant volatiles are likely to play major roles in pea aphid ecology by influencing host plant selection, while aphid-induced plant volatiles released after feeding attract aphid enemies and signal neighboring plants. Yet we know very little about these possibilities especially about differences in volatile profiles among various legume host plants and the effects of feeding by different pea aphid host races on these volatiles profiles. Therefore in this study we used a new low-disturbance method of headspace sampling combined with a mass spectrometry-based non-targeted metabolomic approach (Kallenbach et al., 2014) to identify the volatiles from *M.*

sativa, *P. sativum*, *T. pratense*, and the universal host *V. faba* that might contribute to host choice by various pea aphid host races. Furthermore, we quantified and identified a group of the most regulated volatiles in *M. sativa* plants after infection with these diverse pea aphid host races.

4.2 Materials and Methods

4.2.1 Plant material

For the non-targeted volatile analysis, four legume plant species: *Medicago sativa* cultivar (cv.) 'Giulia' (alfalfa), *Trifolium pratense* cv. 'Dajana' (red clover), *Pisum sativum* cv. 'Baccara' (pea), and *Vicia faba* cv. 'The Sutton' (broad bean), were grown in 7-cm diameter plastic pots with a standardized soil mixture (7:20 mixture of Klasmann Tonsubstrat and Klasmann Kultursubstrat TS1, Klasmann-Deilmann GmbH, Geeste, Germany) in climate chambers maintained at 20 °C, 70 ± 10% relative humidity, and 16 h light/8 h dark photoperiod. *M. sativa* and *T. pratense* plants were used in experiments 20 days after sowing and *P. sativum* and *V. faba* 10 days after sowing.

For the quantification of GLVs, *M. sativa* cultivar 'Giulia' (alfalfa) was grown in 10-cm diameter plastic pots with the same soil mixture and environmental conditions mentioned before.

4.2.2 Aphids

As in the previous research chapters, three pea aphid (*Acyrthosiphon pisum* Harris) clones, each representing one pea aphid host race, were used in the experiments: the clone L84 representing the Medicago race (here called MR), the clone T3-8V1 representing the Trifolium race (TR), and the clone Colmar representing the Pisum race (PR). Aphids were initially collected from their native host plants *T. pratense*, *M. sativa*, and *P. sativum*, respectively, and genotypically assigned to their respective host race (for detailed information see Table S1 in Peccoud (2009)). All aphids were reared on 4-week-old broad bean plants. To synchronize the age of the aphids for the experiments, five apterous female adults were placed on a broad bean plant and were allowed to reproduce for 48 h and were then removed from the plants. Nymphs

were kept on the plants for nine days until they reached adulthood. Then they were transferred to new plants were they reproduced. This procedure was repeated until enough synchronized young adult aphids were available for the experiment. To avoid escape of aphids during the synchronization process, all aphid containing plants were covered with air permeable cellophane bags (18.8 x 39 cm, Armin Zeller, Nachf. Schütz & Co, Langenthal, Switzerland), and placed in a climate chamber under the same conditions described for the plant material.

4.2.3 Volatile collection

For the collection of the plant volatile metabolic profiles, we used silicone laboratory tubing (polydimethylsiloxane (PDMS), 1 mm i.d. x 1.8 mm o.d., Carl Roth) as a low-disturbance method for sampling the headspace. PDMS tubes were prepared in batches, before the experiments, as described (Kallenbach et al., 2014). We used a single batch of PDMS tubes for the experiment.

For headspace sampling of volatile compounds, four clean PDMS tubes were hung on a non-oxidizable wire hook attached to a clean wooden stick placed near each plant. Each plant species was also infested separately with 20 apterous adult aphids from each of the tested clones. The same setup was also used to collect the volatile compounds from plants without aphids. To avoid the escape of aphids, all experimental plants, including aphid-free control plants, were covered with air permeable cellophane bags and were placed in a climate chamber under the same conditions as described above. PDMS tubes were collected 24, 48, 72, and 96 h after aphid infestation and at the start of the experiment, placed in 2 mL amber glass screw-cap vials, and stored at -20 °C for subsequent thermal desorption-gas chromatography-quadrupole mass spectrometry (TD-GC-QMS) analysis. Five replicates were made for each plant-aphid clone combination.

For the sampling of GLV emissions, a dynamic headspace sampling system was used. Individual *M. sativa* plants were placed in 1L glass desiccators (Schott, Jena, Germany) inside a climate chamber under the same conditions as described for the plant material. After plants were infested separately with 20 apterous adult aphids of each pea aphid clone, the desiccators were closed tightly, and the charcoal-purified air was pumped inside at a flow rate of 0.1 L min^{-1}. The volatiles contained in the passive outlet airstream (0.7 L min^{-1}) were collected, 24, 48, 72, and 96 h after aphid infestation and at the start of the experiment, on traps filled with 30

mg of Super-Q adsorbent (Analytical Research Systems, Gainesville, FL, USA). Clean Super-Q traps were used for each collection. The volatiles collected in the Super-Q traps were eluted with 200 µl of hexane and 2 µg of n-nonyl acetate (Sigma-Aldrich, Seelze, Germany) as an internal standard. Eluates were stored in 2 mL glass screw-cap vials at -20 °C for subsequent GC-MS and GC-FID analysis. Four replicates were made for each timepoint for each plant-aphid clone combination.

4.2.4 Volatile analysis

For the analysis of the volatiles collected on the PDMS tubes, a TD-20 thermal desorption unit (Shimadzu) coupled to a GC-MS-QP2010Ultra (Shimadzu) was used. Individual PDMS sampling tubes were placed in 89 mm glass tubes (Supelco) for the desorption of volatiles under a stream of nitrogen at 60 mL min^{-1} for 8 min at 200 °C. Thermally desorbed compounds from the PDMS tubes were first trapped at -20 °C onto a Tenax® adsorbent trap and then heated to 230 °C within 10 s, to be injected on the GC column using helium as the carrier gas (linear velocity: 44.3 cm s^{-1}) and a 10:1 split ratio. The column was a Rtx®-5 GC column (diphenyl dimethyl polysiloxane), 30 m long, with 0.25 mm internal diameter and 0.25 µm film thickness (Restek Corp). The TD-GC interface was held at 250 °C. The GC oven temperature gradient was: 45 °C for 5 min, 6 °C min^{-1} to 260 °C, 100 °C min^{-1} to 300 °C (hold for 3.3 min) with a total run time of 30 min. The transfer line to the detector was kept at 250°C and the ion source at 230 °C. The spectra were recorded by using electron impact ionization (EI) in scan mode at 70 eV from 43 to 350 m/z with a scan speed of 1,666 Da s^{-1}. All data preprocessing and peak integration was performed using the Shimadzu GCMS solutions software (v4.20).

For the chromatographic separation and identification of the GLVs an Agilent 6890 Series GC coupled to an Agilent 5973 N quadrupole mass spectrometer detector was employed. One µl of each sample was injected in splitless mode with an inlet temperature of 220 °C into a Zebron ZB-5 column (5% Phenyl 95% Dimethylpolysiloxane, 30 m x 0.53 mm x 1.50 µm, Phenomenex Inc.), using helium as a carrier gas at 2 ml min^{-1}. The GC oven temperature gradient was as follows: 45°C for 2 min, increased at 6°C min^{-1} to 200°C followed by an increase of 60°C min^{-1} to 300°C and held for 2 min (total run time of 30 min). The parameters for electron impact ionization were as follows: transfer line temperature at 270 °C, repeller 30

V; emission 34.6μA, ionization potential 70 eV, source temperature 230°C, and a scan range of m/z 33–350. Compounds were tentatively identified by direct comparison of spectral data with those from the Wiley and National Institute of Standards (NIST) libraries, and later by comparing their spectral data and retention times with those of commercially available standard compounds.

For quantification of the GLVs, a G1540A gas chromatograph coupled to single flame ionization detector (FID) (HP/Agilent) was employed. For the chromatographic separation, the same conditions, type of column and oven temperature gradient were used as described above. The parameters for the FID detector were as follows: The heater temperature was set at 300°C, hydrogen flow 40 mL min^{-1}, air flow 450 mL min^{-1}, and nitrogen as make-up gas at 45 mL min^{-1}. To determine the concentration of the selected GLVs, the peak areas were compared with those of the internal standard, *n*-nonyl acetate, and concentration calculated using relative response factors and the effective carbon number based on the weight of the compounds (Scanlon and Willis, 1985)

4.2.5 Data analysis

For non-targeted volatile analysis, the retention time correction and peak alignment of the TDU-GC-MS data, an AutoIt (Bennett, 2007) script was used to batch export peak data from the Shimadzu GCMS solutions software (v4.20) as a comma-separated value (.csv) format and then processed by a script written in the Python programming language (Foundation, 2013). Tables with the intensities of the detected features were obtained as output for statistical analysis. All peak intensity tables were uploaded as csv files on the web-based server: MetaboAnalyst (Xia et al., 2009;Xia and Wishart, 2011).

To determine which volatile compounds were unique or shared between given plant species, the sparse Partial Least Squares - Discriminant Analysis (sPLS-DA) algorithm (Lê Cao et al., 2011) was applied to the TDU-GC-MS data collected from uninfested control plants (5 components and 10 variables for each component). A table of the volatile compounds selected in the sPLS-DA model for the 3 first components was generated based on the absolute values of their loadings.

To determine if the pea aphid clones modify the emission of plant volatiles differently over time, a sPLS-DA model was applied to the TDU-GC-MS data collected 24, 48, 72, and 96 h after each plant species was infested with the different aphid races, and uninfested control plants (5 components and 10 variables for each component). Tables for each plant species were generated, with the volatile compounds selected in the 2 first components for each sPLS-DA model, also based on the absolute values of their loadings.

All data from GLVs quantification were analyzed with R version 3.2.0 (R Development Core Team, 2015).

The influence of pea aphid clone and duration of aphid infestation (both used as categorical explanatory variables) on GLVs levels was investigated using a linear mixed effects model (lme from the nlme library (Pinheiro et al., 2015)) with plant ID as random intercept (random = ~1|plantID) to account for the variance heterogeneity of the residuals. The varIdent variance structure was used. Whether the different variance of aphid clones, the duration of aphid infestation or the combination of both factors should be incorporated into the model, was determined by comparing models with different variance structures with a likelihood ratio test and choosing the model with the smallest AIC. The influence (*p*-values) of the explanatory variables was determined with the models with this variance structure by subsequent removal of explanatory variables from the model and comparison of the simpler with the more complex model with a likelihood ratio test (Zuur et al., 2009).

4.3 Results

4.3.1 Legume host plants emit distinct volatile profiles

To determine the general differences in the volatile profiles of the four plant species and select the most discriminative compounds in the data set that separate the samples, the profiles of uninfested control plants of *M. sativa, T. pratense, P. sativum* and *V. faba* uninfested control plants were used in a sparse Partial Least Squares - Discriminant Analysis (sPLS-DA).

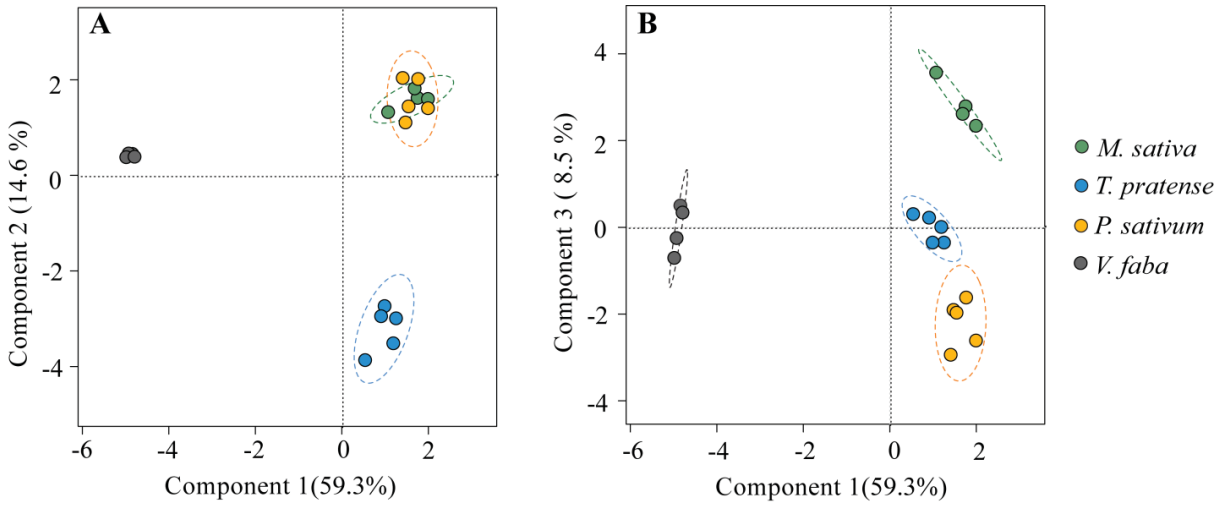

Figure 12. sPLS-DA plots of the headspace volatiles of uninfested control plants. Volatiles were collected with PDMS tubes and analyzed via TDU-GC-QMS. A) Plot with components 1 and 2 B) plot with components 1 and 3. Colored circles represent the volatile profiles of individual plants: green for *M. sativa*, blue for *T. pratense*, yellow for *P. sativum* and black for *V. faba*. Dotted ellipses represent the 95% confidence regions for each group.

The first three components of the sPLS-DA explained 82.4 % of the variance of the data and allowed a differentiation among the species by their volatile profiles (Figure 12). Component 1 (59.3 % of variance) discriminated between *V. faba* and the other three species, while component 2 (14.6 % of variance) separated only the volatile profiles of *T. pratense* plants (bottom) from those from *M. sativa*, *P. sativum,* and *V. faba* (top) (Figure 12A). Component 3 (8.5 %) differentiated particularly *M. sativa* volatile profiles from those of *P. sativum* (Figure 12B).

Table 3. Compounds that discriminate among the uninfested volatile profiles of each species. Each compound is listed with its coefficients for the first three components of the sPLS-DA model in Figure 12. Compounds are offered by their respective retention times (RT).

No.	Name	RT	comp 1	comp 2	comp 3
1	Butanoic acid	3.67			-0.4354
2	1-Hexanol, 2-Ethyl-	9.78			-0.3739
3	2-Octenal	10.54	0.26621		
4	2,3-Octanedione	10.60			0.5694
5	Heptanoic acid	11.02		0.2904	
6	Beta-Linalool	11.62			-0.3507
7	Nonanal	11.74	0.40459		
8	(+)-Nopinone	12.59		0.2201	
9	2-Ethyl-1-hexyl acetate	12.93			0.2058
10	Benzoic acid	13.26		0.2626	
11	Methyl salicylate	14.02		0.6565	
12	Decanal	14.27	0.41692		
13	Benzothiazole	14.74			0.2096
14	2-Ethyl-1-Hexyl propionate	15.08		0.1566	-0.1697
15	2,2-Dimethyl-5-(3-methyl-2-oxiranyl) cyclohexanone	15.37		0.3461	
16	2,4-Dimethylhexan-3-ol	15.44		0.2293	
17	Hexyl benzoate	15.82	-0.4317		
18	1-Decanol	15.85	0.34275		
19	2-Ethyl Hexyl butyrate	17.02			-0.3148
20	3-Hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate	18.15	0.26561		
21	1,1'-Biphenyl	18.29	-0.40039		
22	(Z)-beta-Caryophyllene	19.24	0.12888		
23	(E)-geranyl acetone	19.83	0.17876		
24	(E)-beta-Farnesene	19.93		-0.4082	

In Table 3 we present the loadings with the largest coefficients selected by the sPLS-DA model for the three first components that explained 82.4% of the variability, and helped to separate the profiles of *M. sativa*, *T. pratense*, *P. sativum* and *V. faba* plants in Figure 12. The (-) sign next to the coefficients of the loadings indicate higher abundances of the volatile compounds in the groups positioned on the negative side of each component, while no sign indicates higher abundance in groups positioned on the positive side. For component 1, higher negative coefficients were found for the compounds: hexyl benzoate (No.17; -0.4317) and 1,1'-biphenyl (No.21; -0.4004), which are the primary compounds involved in separating *V. faba* volatile profiles from ones of the other plant species. In the same way, (*E*)-β-farnesene (compound No. 24) had the highest negative coefficient (-0.4082) for component 2 and is mostly responsible for the discrimination of *T. pratense* volatile profiles from those of other plant species. Methyl salicylate (compound No. 11) is the metabolite with the most positive coefficient (0.6565) for the same component, and it helped to discriminate *P. sativum* and *M. sativa* profiles from *T. pratense* volatile profiles. For component 3, the two compounds with the highest positive and negative coefficients: butanoic acid (No.1; -0.4354) and 2,3-

octanedione (No.4; 0.5694) were the variables that most influenced the separation of *P. sativum* and *M. sativa* profiles and from those of *T. pratense* and *V. faba* (Table 3).

4.3.2 Pea aphid host race infestation differentially modified the volatile profiles of legume host plants

To obtain an overview of the effects of pea aphid infestation with different host races on plant volatile profiles, and to choose volatile compounds that help to differentiate them, a sPLS-DA was executed with the complete set of volatile compounds obtained over time from infested and uninfested *M. sativa*, *T. pratense*, *P. sativum* and *V. faba* plants.

For *M. sativa* plants, all biological replicates of the volatile profiles were grouped in an aphid clone-dependent manner in small confidence ellipses, as were the profiles for uninfested control plants (Figure 13). Differentiation among the volatiles induced by different clones was first detected in the sPLS-DA plot for samples collected 48 hours after infestation. The volatile profiles after infestation with the PR clone (right) were discriminated in component 1 (19.6% of variance) from those after infestation with the MR clone (native on *M. sativa*) and uninfested control plants (left). Volatile profiles after infestation with the TR clone (non-native on *M. sativa*) were not well differentiated in this dimension. Component 2 (8.3% of variance) discriminated the volatile profiles generated by the MR clone infestation from those induced by the TR and PR clones and uninfested control plants (Figure 13B). At 72 hours, component 1 (explaining 24.8 % of the variance) separated the volatile profiles of plants infested with the native MR clone (left) from those of the other treatments including uninfested plants (right). Component 2 (10.7 %) discriminated only the volatiles from PR clone-infested plants from those of uninfested control plants, while the other profiles were not differentiated in this dimension (Figure 13C). At 96 hours after aphid infestation, component 1 (7.3 % of the variance) discriminated TR clone-infested plants on the left side of the axis from uninfested control plants on the right of the axis. Component 2 (11.8%) separated the profiles of PR clone (non-native on *M. sativa*)-infested plants (top) from the profiles measured for plants infested with the native MR clone (bottom) (Figure 13D).

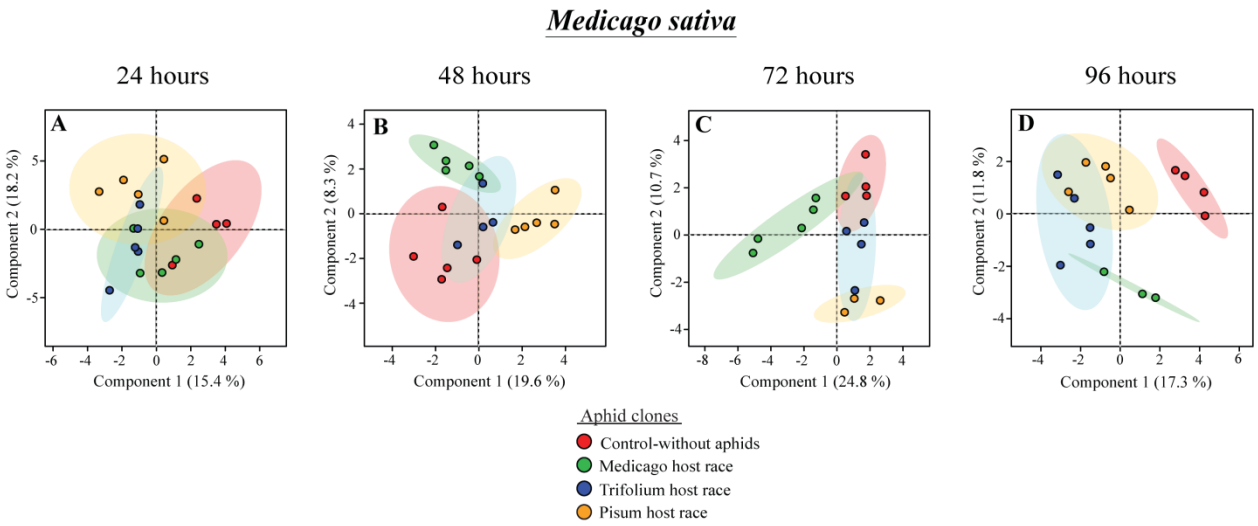

Figure 13.sPLS-DA plots of headspace volatiles of *M. sativa* plants at A) 24, B) 48, C) 72, and D) 96 hours after infestation with different pea aphid clones or left uninfested. Volatiles were collected with PDMS tubes and analyzed via TDU-GC-QMS. Colored circles represent individual plant volatile metabolic profiles after infestation with MR clones (green), TR clones (blue), PR clones (yellow) or uninfested control plants (red). Ellipses represent the 95% confidence regions for each group.

The volatile compounds selected by the sPLS-DA method that helped to differentiate the profiles obtained from infested and uninfested *M. sativa* plants are listed in Table S5. The (-) sign next to the coefficients of the loadings indicate higher abundances of the volatile compounds in the groups positioned on the negative side of each component, while no sign indicates higher abundance in groups positioned on the positive side. At 48 hours the first component of this analysis showed that 3-hexen-1-ol was present at higher amounts in PR clone-treated plants, while other compounds, such as benzothiazole, were more concentrated in the other treatments. For the second component, the C6-volatiles, *n*-hexanal, and 3-hexenal, contribute to the separation and are less abundant after the infestations with the MR clone than after infestations with the TR or PR clones. For the same component, eugenol is only present in uninfested control plants and entirely absent from all plants infested with pea aphid clones. At 72 hours, for the first component, 2,6,11-trimethyldodecane and heptadecane were abundant exclusively in MR clone-infested plants. In contrast, undecanal had lower levels in these plants in comparison with plants from the other treatments. For the second component, 2-undecenal and (*E*)-2-heptenal were two of the volatiles contributing most to the separation on this axis and showed higher levels in plants infested with the PR clone. On the other hand, benzothiazole, which also contributes significantly to this separation, was less abundant in PR clone-infested

plants than in plants from the other treatments. At 96 hours after infestation, for the first component *n*-hexanal, and 3-hexenal were more abundant in uninfested control plants than in aphid-infested ones, as were 2,6,11-trimethyldodecane and heptadecane. In contrast, (*E*)-β-farnesene was more abundant in aphid-infested plants than in uninfested control plants (Table S5).

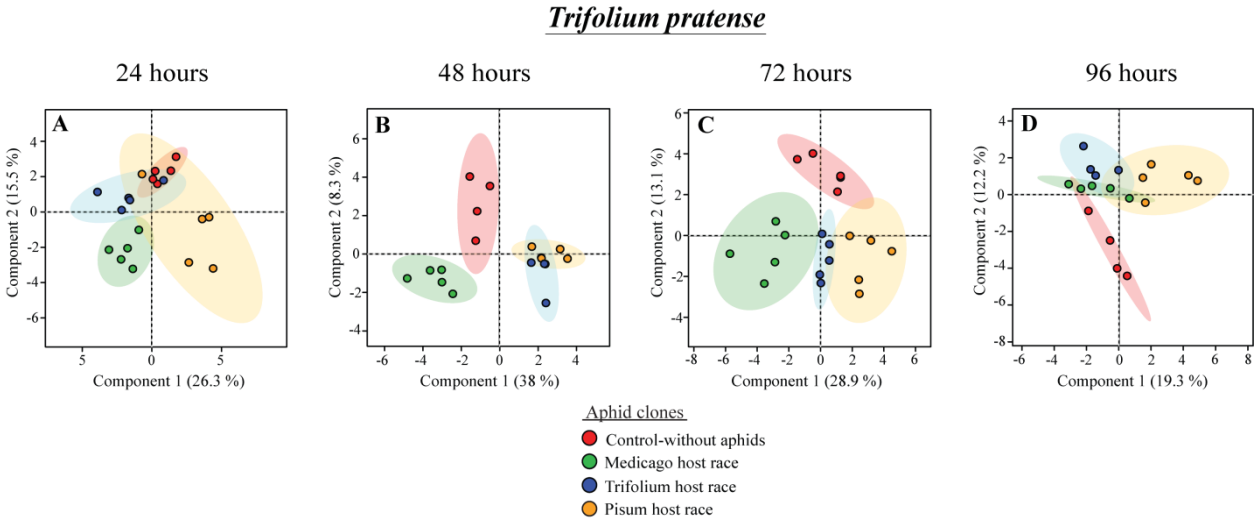

Figure 14. sPLS-DA plots of headspace volatiles of *T.pratense* plants at A) 24, B) 48, C) 72, and D) 96 hours after infestation with different pea aphid clones or left uninfested. Volatiles were collected with PDMS tubes and analyzed via TDU-GC-QMS. Colored circles represent individual plant volatile metabolic profiles after infestation with MR clones (green), TR clones (blue), PR clones (yellow) or uninfested control plants (red). Ellipses represent the 95% confidence regions for each group.

Similarly, in *T. pratense* the volatile profiles were well classified into small groups on sPLS-DA plots depending on whether the plants were infested with specific aphid clones or not infested (Figure 14). However, it was not until 48 hours after aphid infestation that it was possible to obtain good discrimination among the treatment groups (Figure 14B).

The first component of the 48-hour plot explained 38 % of the variance of the volatile metabolite data set and separated MR clone-infested and noninfested control plants (left side of the axis) from plants infested with the PR clone and the native TR clone (right side of the axis) (Figure 14B). The second component, explaining 8.3 % of the variability separated the uninfested control plants from plants infested by the MR and TR clones.

Component 1 (28.9 %) in the 72-hour sPLS-DS plot also separated the volatile profiles of MR clone-infested plants from plants infested with the PR clone. However, the volatile profiles of plants infested with the native TR clone or uninfested were not discriminated by this dimension (Figure 14C). Component 2 (13.1 %) though separates the aphid infested volatile profiles (bottom) from those of uninfested plants (top).

In the 96-hour sPLS-DA plot, the first component explained 19.3 % of the variability of the data and separated the volatile profile of PR clone infestations (right side of the axis) from profiles of other aphid clone infestations and uninfested control plants (left side of the axis) (Figure 14D). As in the 72-hour plot, the second component (12.2 %) also discriminates volatile profiles coming from aphid infested plants (top) from the profiles of uninfested control plants (Figure 14D).

The separation of the different groups in *T. pratense* at 48 hours are determined to some extent by 1-decanol, 1-(2-hydroxy-1-methyl ethyl)-2,2-dimethyl propyl-2-methyl propanoate and 3-hydroxy-2,4,4-trimethylpentyl-2-methyl propanoate for the first component. This volatiles appear in higher levels in MR clone-infested and uninfested control plants than in PR and TR clone-infested plants (Table S6). For the second component, the levels of eugenol were significantly reduced by the infestations with all the aphid clones. A similar pattern was observed for 2,6,11-trimethyldodecane. For the same component, we found again that the C6-volatiles, *n*-hexanal, and 3-hexenal, contributed to the separation, and their levels were reduced after infestation with the MR clone. Conversely, 3-hexen-1-ol amounts were increased after MR and TR clone infestations. After 72 hours, 2-ethyl-1-hexyl propionate, (+)-α-terpineol, and 2- bromooctane are some of the volatiles contributing to the discrimination of the groups. All these metabolites showed a higher abundance in plants infested with the MR aphid clone than in the other treatments. For the second component, nonanal, octanoic acid, and benzothiazole were some of the most discriminant volatiles, with increased levels after all aphid clone infestations. The most discriminating volatiles for the 96-hour sPLS-DA plot were 1-dodecanol, 3-hydroxy-2,4,4-trimethylpentyl-2-methyl propanoate, and 1,3,4-trimethyl-3-cyclohexene-1-carbaldehyde for the first component, with reduced levels after PR clone infestations. In contrast, benzothiazole levels in the same component were only increased by the infestations with the PR clone. For the second component, the levels of 3-hexenal, 2-bromooctane and vanillin were decreased after infestation with all the aphid clones, while 3-hexen-1-ol and

octanoic acid were raised in general for aphid infestations, but most markedly after TR and PR clone infestation (Table S6).

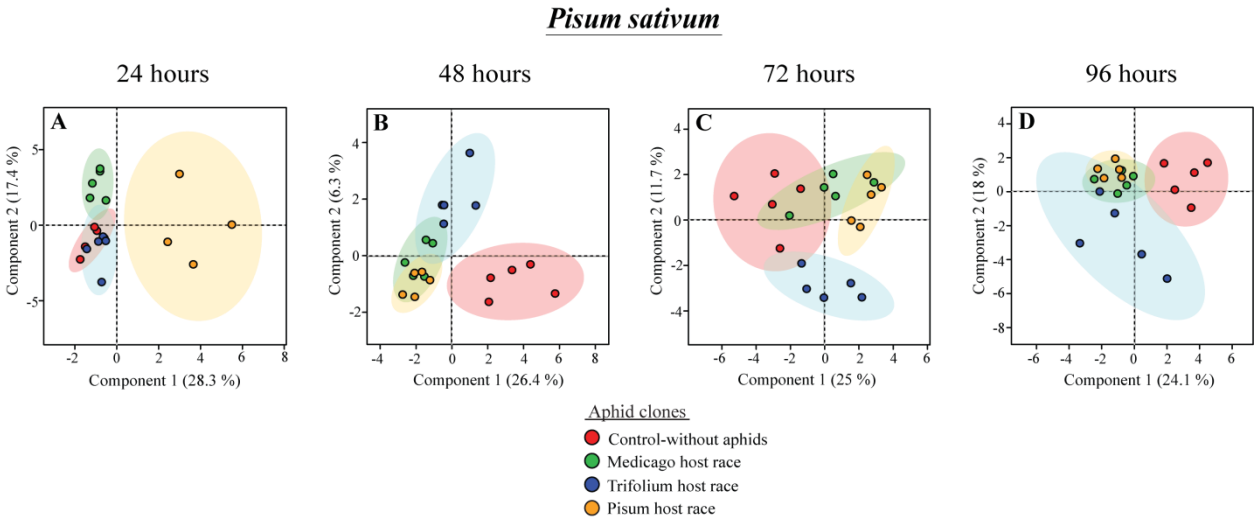

Figure 15. sPLS-DA plots of the headspace volatiles of *P. sativum* plants at A) 24, B) 48, C) 72, and D) 96 hours after infestation with different pea aphid clones and uninfested control plants. Volatiles were collected with PDMS tubes and analyzed by TDU-GC-QMS. Colored circles represent individual plant volatile metabolic profiles after infestation with MR clones (green), TR clones (blue), PR clones (yellow) or uninfested control plants (red). Ellipses represent the 95% confidence regions for each group.

For *P. sativum*, there was significant discrimination of the volatile profiles of different host races in sPLS-DA plots already at 24 hours unlike in *M. sativa* and *T. pratense*, which first showed significant discrimination at 48 hours. The profiles of native PR clone-infested plants (right side of the first component) were well separated from the profiles of the MR and TR clone-infested plants and uninfested control plants (left side of the first component). Component 1 explains 28.3 % of the variability of the total dataset. The second component (explaining 17.4% of the variability) at 24 hours separated the profiles of the MR clone infested plants (top) from the TR clone-infested and uninfested control plants (bottom) (Figure 15A). After 48 hours of infestation, the profiles of the native PR clone overlapped with those of the non-native MR clone (Figure 15B, C, and D). At 48 hours discrimination was also seen in component 1 (26.4 % of variance) between the MR and PR clone-infested plants (left) and uninfested control plants (right) (Figure 15B). Component 2 (6.3 % of the variance) separated the profiles of TR clone infestations (top) from those of PR clone infestations and uninfested control plants.

Some *P. sativum* volatile compounds contribute to the separation of the native PR clone-infested plants from the other treatments at 24 hours. From the first component, 2-butyl-1-octanol and eugenol are only emitted by plants infested with the PR clone, while (+)-α-terpineol was significantly reduced by infestation with the same clone. From the second component, the levels of the C9 aldehydes (*E*)-2-nonenal and nonanal as well as the C10- aldehyde decanal dropped after MR and PR clone infestations (Table S7). After 48 hours, the amounts of terpinen-4-ol, 2-ethyl-1-hexanol, and 2-bromooctane were reduced by infestations with all aphid clones, and in the second component, the metabolites (*E*)-β-farnesene and methyl salicylate displayed higher levels when the TR clone fed on. At 72 hours, although the abundance of 2-bromooctane, 3-hydroxy-2,4,4-trimethylpentyl 2-methyl propanoate, and 1-decanol were reduced by all aphid clones (component 1), with reduction most marked after infestation with the native PR aphid clone. (+)-α-Terpineol levels were also decreased by all aphid clones (component 2). However, the strongest reduction was observed when the TR clone fed. Conversely, after the infestation of the TR clone the levels of the volatiles 1, 4-diacetylbenzene, vanillin, and nonanoic acid increased. At 96 hours, the most discriminating compounds for the first component were the C7 aldehyde, (*E*)-2-heptenal; the C9 aldehydes, (*E*)-2-nonenal and nonanal; and the C10 aldehyde, decanal. Levels of these aldehydes were increased by infestation with all the aphid clones. For the second component, 1-decanol; 6-undecanone; (*3Z*)-4,8-dimethyl-3,7-nonadien-2-one; and (+)-α-terpineol were observed in higher amounts when the TR clone fed (Table S7).

At 72 hours on *P. sativum*, component 1 (25 % of the variance) divided the volatile profiles from native PR clone infested plants (right) from those from uninfested control plants. Equally important, component 2 (11.7 %) separated the volatile profiles of TR clone-infested plants (bottom) from the profiles obtained from the infestations with the other clones and uninfested control plants (top) (Figure 15C). At 96 hours, none of the components discriminated among treatments. However, an overall separation of the profiles of aphid-infested plants from those of uninfested plants was observed (Figure 15D).

In contrast to the discriminations observed for the treatments in the other plant species, aphid clone treatments on the 'universal host', *V. faba*, did not show much discrimination (Figure 16). Only in the sPLS-DA plot for 48 hours did the treatments display some discrimination (Figure 16B). Additionally, infestation treatments were sometimes differentiated

from uninfested control plants. e.g., the MR clone infestation at 24 and 96 hours (component 1, 34% and 27% of the variance, respectively), and the PR clone at 48 hours (component 1, 29.4 %) (Figures 15A-C). At 96 hours all treatments overlapped in the center of the plot showing no discrimination among the treatments (Figure 16D). Volatile compounds that contributed to the minor discrimination among the treatments in at least three of the time points included 2-ethyl-1-hexyl acetate, (-)-borneol, octanoic acid, terpinen-4-ol, (+)-α-terpineol, and 2-ethyl-1-hexyl propionate (Table S8).

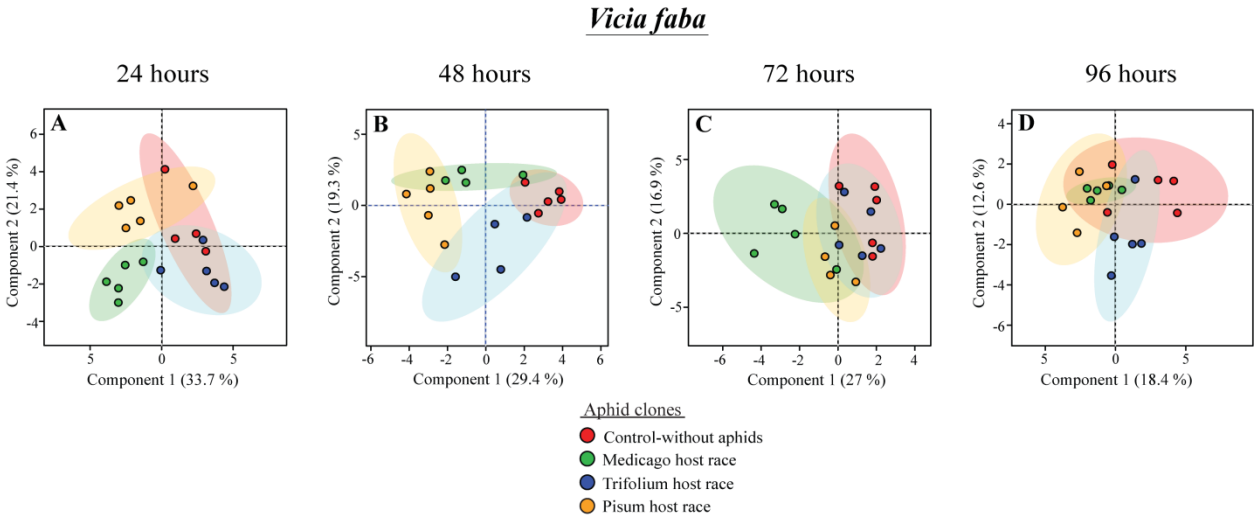

Figure 16. sPLS-DA plots of the headspace volatiles of *V. faba* plants at A) 24, B) 48, C) 72, and D) 96 hours after infestation with different pea aphid clones and uninfested control plants. The volatiles were collected on PDMS tubes and analyzed by TDU-GC-QMS. Colored circles represent individual plant volatile metabolic profiles after infestation with MR clones (green), TR clones (blue), PR clones (yellow) and uninfested control plants (red). Ellipses represent the 95% confidence regions for each group.

4.3.3 The native MR clone induced lower levels of GLVs in *M. sativa* plants than clones of non-native races

Based on the importance of GLVs for the strong separations of the treatments on *M. sativa* and *T. pratense* plants observed at 48 hours, we quantified the levels of the main three GLVs, (*Z*)-3-hexenal, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate in volatile collections made with a dynamic headspace sampling system of *M. sativa* plants separately infested with each of the aphid clones and in uninfested control plants.

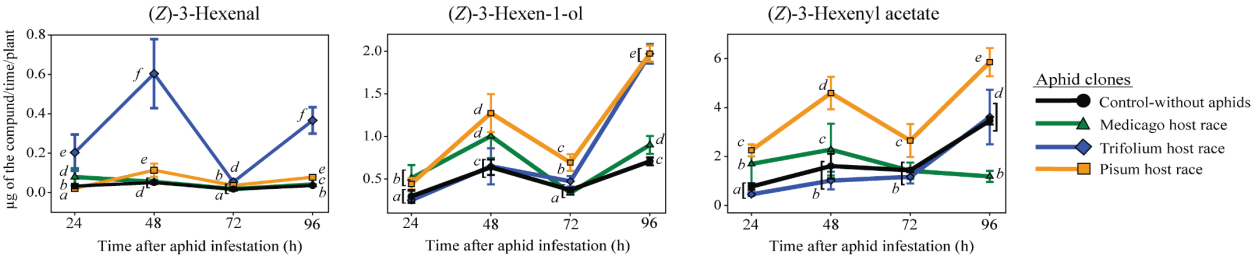

Figure 17. Amounts of the green leaf volatiles A) (Z)-3-hexenal; B) (Z)-3-hexen-1-ol and C) (Z)-3-hexenyl acetate emitted from *M. sativa* plants after infestation with pea aphid clones of different host races. Symbols represent means ± SE. Statistical values are presented in Table S9. Significant differences ($P \leq 0.05$) between aphid clones at different time points are indicated by different letters.

The amount of (*Z*)-3-hexenal emitted from *M. sativa*-infested plants varied with the aphid clone used but was always higher at all time points than the levels measured from uninfested control plants over time (Figure 17A). The emission of this GLV compound by plants infested with the non-native TR and PR clones were usually significantly greater than by plants infested with the native MR clone, except at 24 hours. In contrast, (*Z*)-3-hexenal emissions from plants infested with the native MR clone were low, constant and equivalent to those emitted by the uninfested control plants over the entire experiment (Figure 17A).

The emissions of the other two GLVs, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate from aphid-infested plants were greater or equivalent to those emitted from uninfested control plants (Figure 17B and C). As for (*Z*)-3-hexenal, emission of these two compounds after infestation with the native MR clone was significantly less than following the non-native TR and PR clones after 72 hours. However, at earlier time points, the emission from infestation with the MR clone was higher than that from the TR-infested clone.

4.4 Discussion

4.4.1 Pea aphid host plant species emit diferent volatile bouquets.

Analysis of the volatile profiles of the four legume species studied without aphid treatment revealed that each species possesses its own distinct mixture. The volatile composition of the headspace of the universal host plant, *V. faba,* is different from the others

(Figure 12), containing much higher amounts of hexyl benzoate and 1,1'-biphenyl than in the other plant species. Conversely, the C8, C9, and C10 aldehydes together with C10 Alcohols and two terpenoid compounds are present at higher levels in the other species than in *V. faba* (Table 3, component 1). These volatile compounds could act as cues for the pea aphid clones to distinguish the universal host from the other less suitable host plants (Gen-ichiro et al., 2016).

Among the other species, the volatile blends of *M. sativa* and *P. sativum* contained much higher amounts of methyl salicylate. This compound may act as a repellent volatile for the black bean aphid, *Aphis fabae* (Hardie et al., 1994), and so the high constitutive methyl salicylate content of *M. sativa* and *P. sativum* might repel the attack of most pea aphid host race clones. Conversely, *T. pratense* contains significantly higher levels of (*E*)-β-farnesene than *M. sativa* and *P. sativum*. (*E*)-β-farnesene is well known as an alarm pheromone in aphids (Edwards et al., 1973) released as a response to threats, such as predation, and leading to dispersal. Although constitutive emission of (*E*)-β-farnesene could repel aphids (Gibson and Pickett, 1983), the responses of aphids to this compound are very variable and depend on the host plant (Jing-Gong et al., 2002) and the aphid species (Montgomery and Nault, 1977;Kunert et al., 2010). (*E*)-β-farnesene also serves plants as an indirect defense mechanism against aphids by attracting their parasitoids. For example, Micha and Wyss (1996) reported that (*E*)-β-farnesene is a host finding kairomone of the aphid parasitoid *Aphidius uzbekistanicus*. In contrast, other studies showed that this compound is not a cue for aphid predators like lacewings (*Chrysoperla carnea*) and ladybirds (*Coccinella septempunctata*) (Joachim and Weisser, 2015). Thus, the high constitutive (*E*)-β-farnesene levels emitted from *T. pratense* could act as a repellent for some pea aphid clones or as a signal to attract aphid parasitoids.

The volatile profile of *M. sativa* is differentiated from that of *P. sativum* by the amounts of C6-aldehydes and -alcohols derivatives compounds, 2,3-octanedione, benzothiazole, butanoic acid, and linalool. When the monoterpene linalool is released from damaged lima bean leaves, it can induce and prime indirect defense in neighboring undamaged plants (Heil and Silva Bueno, 2007). Linalool has also been reported to have insecticidal, and repellent activity against the cotton aphid (*Aphis gossypii*) (Jiang et al., 2016), and therefore could be acting as a direct and indirect defense against aphids in *M. sativa* and *P. sativum* plants.

4.4.2 Native pea aphid host races reduce the emission of toxic volatile compounds

Chapters 1 and 2 of this thesis described how infestation with various pea aphid host races caused distinct changes in the defense hormones and other defense secondary metabolites of their host plants. Here host races were also found to cause distinct changes in the volatile profiles of infested plants. Although it was possible to observe some aphid-induced changes in plant volatile profiles after only 24 hours of infestation, it was not until 48 hours that significant differences were apparent for *M. sativa* and *T. pratense.* This timescale though is consistent with the significant differences in host race performance seen after 48 hours of feeding on these plant species (Sanchez-Arcos et al., 2016). Therefore, we centered our attention on the chemical differences between treatments at 48 hours.

The infestation of all pea aphid clones caused a substantial reduction in the levels of eugenol emitted at 48 hours from *M. sativa* and *T. pratense*. Eugenol is a common constituent of commercial insecticides used against aphids, spider mites, and whiteflies (Cloyd et al., 2009), and has shown high repellence and toxicity against nymphs of the green peach aphid, *Myzus persicae* (Isman, 2000) and adults of the cowpea aphid, *Aphis craccivora* (Tewary et al., 2006). The reduction of eugenol emission by all the pea aphid clones might point to a general aphid mechanism to reduce the toxicity of their food plants. However, there was no reduction of eugenol emission by aphids feeding on *V. faba*, and in *P. sativum* eugenol emission is also triggered by the native PR clone. Thus the different host races may employ different strategies to manipulate plant defenses.

Benzothiazole also contributed to the discrimination of the volatile blends induced by pea aphid infestation on all plants except *V. faba*. The emission of this heterocyclic aromatic compound was especially influenced by an infestation of the PR clone, which reduced the levels of benzothiazole at early time points but increased it at 96 hours. There is not much evidence about plant sources and biological activities of benzothiazole, but this compound has been described to be emitted by *A. thaliana* with potential activity in plant-insect interactions and plant defense (Rohloff and Bones, 2005). Benzothiazole also has been reported to have a signaling role between poplar trees (Hu et al., 2009;Tang et al., 2013), and rice plants infested with the rice stinkbug released a greater amount of benzothiazole than uninfested plants (Melo Machado et al., 2014). Benzothiazole was also shown to have adverse effects on the Chinese

chive maggot, *Bradysia odoriphaga* (Zhao et al., 2016). To the best of our knowledge, there are no previous studies on the relationships between pea aphids and this volatile compound. However, the fact that the PR clone has a specific ability to modulate benzothiazole emission indicates once more that on a chemical level plants respond differently to different pea aphid clones.

The monoterpenoids terpinen-4-ol and (+)-α-terpineol contributed significantly to the discrimination of volatile profiles after infestation on *P. sativum* and *V. faba* plants. In general, their emission was mostly reduced by the infestation of pea aphid clones, except at 96 hours in *P. sativum* (Tables S7 and S8). As common components of plant essential oils, the activities of terpinen-4-ol and (+)-α-terpineol have been tested against many insects. For example, both volatiles extracted from the essential oil of *Melaleuca alternifolia* showed antifeedant activities against the cotton bollworm, *Helicoverpa armigera* (Liao et al., 2017). Similarly, a fraction of the essential oil of *Artemisia rupestris* containing both monoterpenes had insecticidal and repellent activity against the booklice, *Liposcelis bostrychophila* (Liu et al., 2013). Terpinen-4-ol from the essential oils of rhizomes of *Alpinia conchigera*, *Zingiber zerumbet*, and *Curcuma zedoaria* had repellent activity against *Sitophilus zeamais* and *Tribolium castaneum* (Suthisut et al., 2011). Most relevant for our study is the evidence of activities on aphids. For example, α-terpineol has been reported to repel the aphid *Myzus persicae* even before it lands on the host plant (Hori, 1998). The essential oil of *Lavandula angustifolia*, which is rich in terpinen-4-ol, caused a high mortality of the pea aphid (Attia et al., 2016). Therefore, the tendency of all tested clones to reduce the emission of these monoterpenes in our study suggests that this may be the result of specific aphid behavior to reduce the defenses of the plant. On the other hand, the twelve-spotted lady beetle, *Coleomegilla maculataan* a predator of *A. pisum* showed a high antennal response to (+)-α-terpineol and was attracted to traps baited with this volatile (Zhu et al., 1999). Thus, another critical reason for aphids to reduce the emission of these monoterpenes is to reduce the risk of predator attraction.

The major group of volatile compounds that discriminated among treatments in this study included C6-C10 aldehydes, alcohols, and derivatives. Within this group are the green leaf volatiles (GLVs): the C6 compounds *n*-hexanal, (*Z*)-3-hexenal, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate, known for their roles as defense signals in plants. The aldehydes *n*-hexanal and (*Z*)-3-hexenal are the initial GLVs formed by cleavage of fatty acid hydroperoxides. These

compounds are well known to decrease aphid fecundity (Hildebrand et al., 1993), and aphids feeding on transgenic plants with reduced levels of *n*-hexanal and (*Z*)-3-hexenal displayed a two-fold increase in fecundity (Vancanneyt et al., 2001). In our study, the MR pea aphid clone reduced the levels of n-hexanal and (*Z*)-3-hexenal particularly on *M. sativa* (the native host) and *T. pratense* plants (Table S5 and S6), which could be a result of a deliberate strategy to minimize the levels of these toxic metabolites. In fact, the performance of the MR clone on *M. sativa* was found be significantly better than on other hosts (Sanchez-Arcos et al., 2016) (Chapter 1). However, in a previous study (*Z*)-3-hexenal was found not to differ between *A. pisum*-infested and uninfested *M. sativa* plants (Pareja et al., 2009). However, in the same study, the levels of this volatile were found to be reduced in *A. pisum*-infested vs. uninfested *V. faba* plants.

Of all the GLVs, (*Z*)-3-hexen-1-ol has the strongest adverse effects on aphid fecundity (Hildebrand et al., 1993), along with other roles in herbivore repellence and attraction, the attraction of herbivore enemies, and induction of defenses in uninfested plants (Wei and Kang, 2011). In our study, the MR and TR clones decreased the emissions of (*Z*)-3-hexen-1-ol to levels comparable to those of uninfested plants, although not at all time points (Table S5 and S6), which could prevent a decrease in fecundity.

Unlike the other GLVs tested, (*Z*)-3-hexenyl acetate, although reported before as a volatile from *M. sativa* plants (Blackmer et al., 2004), did not reduce aphid fecundity when tested at similar levels as *n*-hexanal and (*Z*)-3-hexenal (Hildebrand et al., 1993). This may explain why this volatile was not generally reduced by aphid infestations (Table S5).

4.4.3 Reduction of GLV emissions by native pea aphid host races minimizes direct and indirect plant defenses and plant defense signaling

Due to the major role played by GLVs in discriminating among the volatile blends released from aphid-infested plants, we carried out additional headspace collections with a dynamic system to quantify the levels of GLVs for different treatments of *M. sativa* plants. The results showed again that native pea aphid host races reduce the emission of GLVs on their host plants more than non-native host races, and may help rationalize their better performance (Chapter 1).

A reduction in plant GLV emission might benefit aphids several different ways: 1) by reducing the direct toxic or deterrent effects, 2) by minimizing the attraction of aphid predators and parasitoids (indirect defense), and 3) by reducing the possible priming of defenses in other parts of the plant or in neighboring plants. Evidence for the first scenario is limited since we know only that GLVs reduce the fecundity of the tobacco aphid (*Myzus nicotianae*) (Hildebrand et al., 1993), and that reduction in GLV emission by transgenic knock-down of the hydroperoxide lyase branch of oxylipin metabolism in transgenic potato plants increases the fecundity of the green peach aphid *Myzus persicae* (Vancanneyt et al., 2001). However, GLVs elicited high electroantennogram responses in *A. pisum* (van Giessen et al., 1994), which could be a mechanism to avoid this toxin or behavioral deterrent.

Much evidence demonstrates the role of GLVs in attracting predators or parasitoids of aphids. The electroantennographic (EAG) response of the marmalade hoverfly, *Episyrphus balteatus*, a well-known aphid-specific predator, to GLVs was stronger than to other volatiles, including terpenoids and insect pheromones (Verheggen et al., 2008). Similarly robust EAG responses were elicited by GLVs on two *A. pisum* predators, the twelve-spotted lady beetle, *Coleomegilla maculata* and the green lacewing, *Chrysoperla carnea* (Zhu et al., 1999). Further, (*Z*)-3-hexenyl acetate emitted from *Myzus persicae*-infested *A. thaliana* genotypes was a predominant attractant for the parasitic wasp *Aphidius colemani* (Chehab et al., 2008). All these suggest that suppression of GLV emission might be a mechanism by aphids to limit the plant's ability to attract aphid predators and parasitoids.

The third scenario for how the reduction of GLV emission could benefit aphids concerns the possibility that GLVs act to stimulate defenses in unattacked parts of the plant or neighboring plants. For example, exposing maize seedlings to GLVs emitted from neighboring plants led to significant increases in JA, sesquiterpenes, and GLVs (Engelberth et al., 2004;Yan and Wang, 2006). GLVs also serve as signals to prime defenses in *A. thaliana*, but these compounds must be emitted intermittently and at low concentrations to have an optimum effect (Shiojiri et al., 2012). Such metabolite changes affect aphids as shown by the fact that *Myzus persicae*, for example, was no longer attracted to potato plants after they had been exposed to a volatile blend containing GLVs (Dahlin et al., 2015). Thus the reduction of the GLVs by aphid infestation might suppress the ability of host plants to induce or prime defense induction in adjacent host plants or plant parts.

Each of these scenarios provides a rationale (improved host quality, reduction of enemies, improved quality of future food sources in the vicinity) for the observed suppression of GLV release by pea aphids on their native host plants. However, much additional experimentation is necessary to fully understand the effects of aphids on GLVs and the effects of GLVs on plants and aphid enemies.

5 Concluding discussion

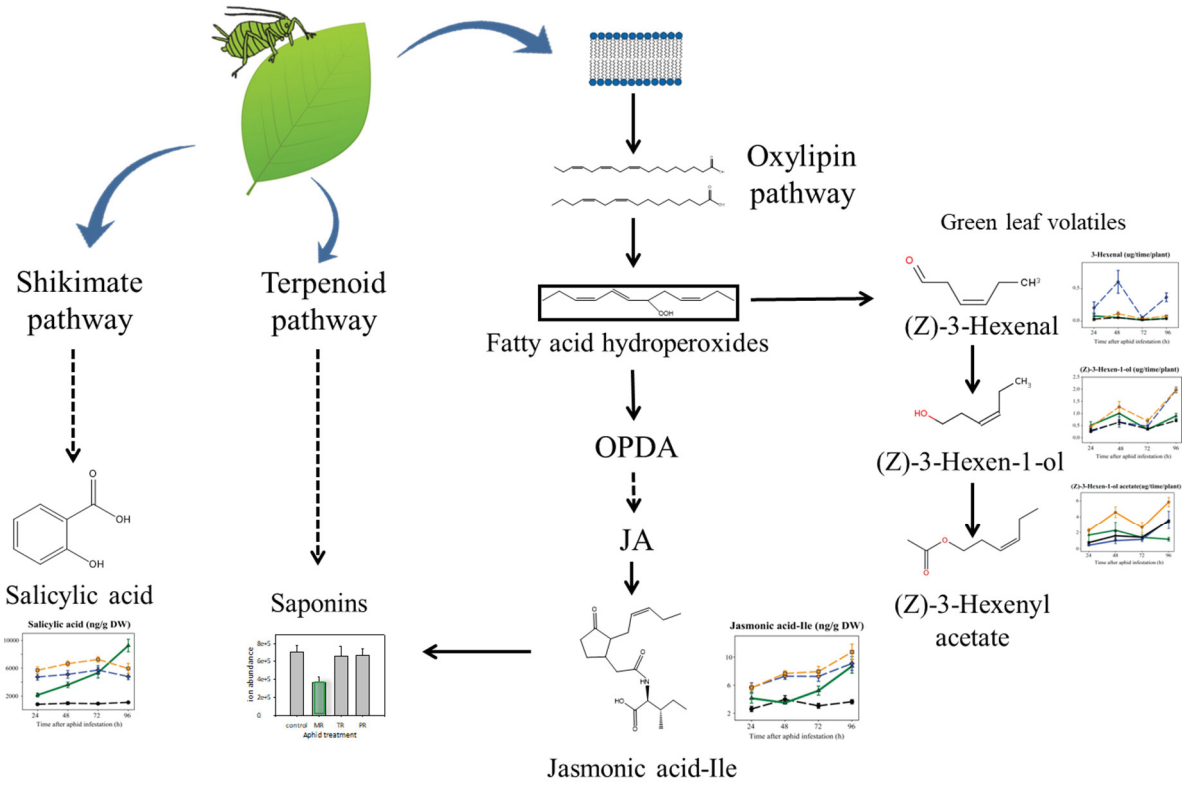

Figure 18. Overview of the main results obtained during this thesis concerning the ability of native pea aphid host races (such as that found on *M. sativa*) to reduce the levels of defense hormones on their native hosts, which in turn reduces other defensive metabolites such as saponins and GLVs. cis-(+)-12-Oxo-Phytodienoic Acid (OPDA), jasmonic acid (JA).

In this work we have shown that the ability of native host races of the pea aphid complex to feed and perform well on their specific legume host plant is correlated with their capacity to modulate the central plant defense signaling pathways through reduction of SA and JA-Ile levels, which leads to a reduction in non-volatile and volatile plant defense metabolites, like saponins, flavonoids, and GLV (Figure 18). Strikingly, the reductions in defense hormones and other defense metabolites generated by native aphid race feeding occurred even though plant damage on native hosts was much higher than on non-native hosts as a consequence of greater aphid abundance resulting from higher growth, survival and reproduction rates. Since the reduction of levels of the active JA-Ile conjugate correlated with reductions in the levels of other JA pathway metabolites (OPDA, JA, and hydroxylated and carboxylated JA and JA-Ile

derivatives), and with reductions in the levels of GLVs, native aphid host races most likely are able to suppress both major branches of the oxylipin pathway, the allene oxide synthase (AOS) branch to the jasmonates and the hydroperoxide lyase (HPL) branch to the GLVs (Savchenko et al., 2013) by blocking an upstream step. Suppression of the oxylipin pathway reduces the activation of direct defense responses by jasmonates and the signaling of herbivore enemies and neighboring plants by GLVs (Chehab et al., 2008).

Although the non-targeted and targeted metabolomic approaches used during this work revealed the capacity that native pea aphid clones have to modulate many aspects of plant metabolism, further work is necessary to identify how these defense mechanisms are regulated concerning aphid signals and to each other. Thus transcriptomic and proteomic studies must be performed to elucidate changes in gene expression and protein abundance after infestation with the various pea aphid clones and identify possible points of regulation in each pathway. Additional research is also needed to understand the chemical effectors employed by pea aphids to reduce defense signals. Previous aphid work has focused on salivary effector proteins that are injected into host plants and the way these can modulate plant processes to facilitate feeding (Hogenhout and Bos, 2011;Pitino and Hogenhout, 2013). Since all pea aphid clones, both native and non-native, are able to begin penetrating the plant (Schwarzkopf et al., 2013), but only some can feed and perform well, the type and quantity of these effectors may be critical in modulating plant defense signaling and mediating aphid success. Future work on the nature of these effectors and the differences among pea aphid host races may help identify the basis for differential defense signaling.

Future work is also needed to investigate the connection between defense signaling cascades and defense metabolite accumulation in legumes. Studies on plants from other families indicate that reductions in defense hormones lead to a reduction in defense metabolite formation. For example, jasmonates can elicit the accumulation of saponins in cell cultures and intact plants (Hayashi et al., 2003;Hu and Zhong, 2007;2008;Rahimi et al., 2015;Rahimi et al., 2016a) by stimulating the activity of common LOX enzymes (Rahimi et al., 2016b) or inducing the transcription of terpene biosynthetic enzymes like squalene synthase (SS), squalene epoxidase (SE), and β-amyrin synthase (β-AS) (Suzuki et al., 2002) relevant to saponin biosynthesis (Figure 18). Moreover, Both SA and JA pathways can promote the biosynthesis of flavonoids in plants (Dombrecht et al., 2007;Sun et al., 2016). Hence by reducing defense

hormone levels in legumes, native host races may also be able to reduce the toxicity and deterrence of the host plant by reducing compounds like saponins or flavonoids.

Not all plant hormone pools were influenced by pea aphid infestation in this study. For example, ABA concentrations were not affected by the native or non-native status of the infesting aphid clone indicating that ABA does not make a significant contribution to the differential ability of pea aphid host races to colonize and feed on a plant. In future work, the levels of other hormones such as auxins, cytokinins, gibberellins, ethylene, and brassinosteroids, should be quantified to assess their role in triggering legume defenses after aphid infestation.

Finally, the methods used in this work demonstrated the potential that targeted and non-targeted metabolomic approaches have to understand the chemistry of plant-aphid interactions. We conducted a targeted analysis of defense hormones since SA and JA are common to all seed plants. However, due to our relative ignorance of phloem-localized defense systems, it is not possible in advance to suggest which other types of compounds might be modulated by aphid infestation. Thus, non-targeted methods were valuable in identifying, for example, saponins as potential defense compounds in this interaction. Other aspects of our analytical platform also contributed to the success of our work. For example, given the small size of aphids and difficulties in rearing them, the samples of infested plant tissue analyzed were always small, requiring the use of sensitive mass spectrometric detection methods for both LC and GC separations. In addition, because of the need to investigate many aphid clone-plant combinations to understand the basis of aphid host race specificity, a large number of samples must be analyzed requiring highly automated instrumentation and bioinformatics tools as we were able to employ.

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7 Supplementary Material

Table S1: Statistical values for the analysis of the survival of adult aphids on different legume species according to aphid clone, time of aphid infestation, and the interaction between aphid clone and time of aphid infestation.

Plant species	Statistical test used	Factor	F/*Deviance*	*P*-value
M. sativa	glm/quasibinomial	Interaction	9.393	**<0.001**
		Clone	61.897	**<0.001**
		Time	39.620	**<0.001**
T. pratense	glm/quasibinomial	Interaction	2.201	0.121
		Clone	32.077	**<0.001**
		Time	17.905	**<0.001**
P. sativum	glm/quasibinomial	Interaction	9.402	**<0.001**
		Clone	29.848	**<0.001**
		Time	36.724	**<0.001**
V. faba	glm/binomial	Interaction	*–0.774*	0.679
		Clone	*–0.457*	0.796
		Time	*–21.990*	**<0.001**

Significant P-values are given in bold. Depending which statistical test was used F-values or Deviance are given. Deviance values are given in italics.

Table S2: Statistical values for the analysis of the weight of surviving adult aphids on different legume species according to aphid clone, time of aphid infestation, and the interaction between aphid clone and time of aphid infestation.

Plant species	Statistical test used	Factor	F/*L-ratio*	*P*-value
M. sativa	ANOVA	Interaction	2.105	0.072
		Clone	30.790	**<0.001**
		Time	24.190	**<0.001**
T. pratense	ANOVA	Interaction	1.722	0.137
		Clone	152.140	**<0.001**
		Time	36.520	**<0.001**
P. sativum	ANOVA	Interaction	2.841	**0.019**
		Clone	24.307	**<0.001**
		Time	37.734	**<0.001**
V. faba	gls/varIdent error structure for each time-clone combination	Interaction	*35.768*	**<0.001**
		Clone	*24.540*	**<0.001**
		Time	*28.487*	**<0.001**

Significant P-values are given in bold. Depending which statistical test was used F-values or Likelihood ratios are given. Likelihood ratios are given in italics.

Table S3: Statistical values for the analysis of the total weight of offspring produced on different legume species according to aphid clone, time of aphid infestation, and the interaction between aphid clone and time of aphid infestation.

Plant species	Transformation	Factor	*F*-value	*P*-value
M. sativa	sqrt	Interaction	3.655	**0.005**
		Clone	67.914	**<0.001**
		Time	19.396	**<0.001**
T. pratense	sqrt	Interaction	3.936	**0.003**
		Clone	84.247	**<0.001**
		Time	42.997	**<0.001**
P. sativum	sqrt	Interaction	5.113	**<0.001**
		Clone	28.904	**<0.001**
		Time	216.371	**<0.001**
V. faba	sqrt	Interaction	7.479	**<0.001**
		Clone	66.321	**<0.001**
		Time	481.858	**<0.001**

Significant P-values are given in bold.

Table S4: Statistical values for the analysis of phytohormone levels in different legume species according to aphid clone, time of aphid infestation, and the interaction between aphid infestation.

Phytohormone	Factor	*Medicago sativa*			*Trifolium pratense*			*Pisum sativum*			*Vicia faba*		
		Variance	*L*-ratio	*P*-value	Variance	*L*-ratio	*P*-value	Variance	*L*-ratio	*P*-value	Variance	*L*-ratio	*P*-value
SA	Interaction	Treat	39.153	**<0.001**	Treat	50.454	**<0.001**	Treat	36.765	**<0.001**	Treat	27.395	**0.001**
	Time		7.336	0.062		8.873	**0.031**		1.682	0.641		47.924	**<0.001**
	Clone		222.283	**<0.001**		52.913	**<0.001**		15.691	**0.001**		7.499	0.058
JA	Interaction	Treat	25.652	**0.002**	Treat	46.036	**<0.001**	Treat	59.924	**<0.001**	Treat	46.836	**0.001**
	Time		35.843	**<0.001**		2.629	0.452		17.347	**<0.001**		41.140	**<0.001**
	Clone		58.841	**<0.001**		84.842	**<0.001**		11.623	**0.009**		10.147	**0.017**
ABA	Interaction	Treat	29.659	**<0.001**	Race	25.066	**0.003**	Time	31.306	**<0.001**	Time	12.635	0.180
	Time		24.361	**<0.001**		21.067	**<0.001**		9.962	**0.019**		22.568	**<0.001**
	Clone		10.416	**0.015**		61.095	**<0.001**		63.165	**<0.001**		5.243	0.155
OPDA	Interaction	Treat	83.702	**<0.001**	Treat	40.459	**<0.001**	Treat	33.652	**<0.001**	Treat	61.859	**<0.001**
	Time		41.687	**<0.001**		3.666	0.300		20.965	**<0.001**		58.519	**<0.001**
	Clone		75.721	**<0.001**		51.441	**<0.001**		16.154	**0.001**		10.430	**0.015**
JA-Ile	Interaction	Treat	36.223	**<0.001**	Treat	15.788	0.071	Time	23.719	**0.005**	Treat	26.854	**0.002**
	Time		17.566	**<0.001**		19.783	**<0.001**		17.909	**<0.001**		34.264	**<0.001**
	Clone		99.135	**<0.001**		78.228	**<0.001**		11.373	0.251		8.941	**0.030**
OH-JA1	Interaction	Treat	29.956	**<0.001**							Time	37.378	**<0.001**
	Time		45.957	**<0.001**		Not detectable			Not detectable			13.691	**0.003**
	Clone		49.960	**<0.001**								11.000	**0.012**
OH-JA2	Interaction	Treat	76.052	**<0.001**	Treat	19.502	**0.021**				Treat	37.372	**<0.001**
	Time		7.005	0.072		26.260	**<0.001**		Not detectable			34.891	**<0.001**
	Clone		117.077	**<0.001**		91.853	**<0.001**					1.158	0.763
OH-JA-Ile	Interaction	Treat	82.928	**<0.001**	Race	10.144	0.339				Treat	13.692	0.134
	Time		12.537	**0.006**		2.353	0.502		Not detectable			28.695	**<0.001**
	Clone		23.852	**<0.001**		53.008	**<0.001**					9.006	**0.029**
COOH-JA-Ile	Interaction	Treat	29.033	**<0.001**	Treat	27.277	**0.001**						
	Time		4.712	0.194		7.143	0.068		Not detectable			Not detectable	
	Clone		32.150	**<0.001**		1.808	0.613						

To account for the variance heterogeneity of the residuals the varIdent variance structure was used. Under "Variance" it is specified whether the variance is controlled for each aphid clone-plant species combination (Treat), for each aphid race (Race) or for each time point (Time). Significant P-values are given in bold.

Table S5. Volatile compounds (with their coefficients) selected from the first two components of each sPLS-DAs of volatile metabolic profiles *M. sativa* plants after 24, 48, 72 and 96 hours of infestation with different pea aphid clones and uninfested plants (Figure 13). volatiles are sorted by their retention time (RT). For each component, only the loadings with the largest coefficient are presented. (+) and (-) next to the loadings indicate if they are positive and negative more abundant.

			24 hours		48 hours		72 hours		96 hours	
No	Compound name	RT	comp 1	comp 2	comp 1	comp 2	comp 1	comp 2	comp 1	comp 2
1	3-Methyl-2-buten-1-ol	3.49								-0.5457
2	n-Hexanal	3.98				-0.4449			0.2798	
3	3-Hexenal	5.16				-0.5517			0.5022	
4	3-Hexen-1-ol	5.52			0.7538					
5	(E)-2-Heptenal	7.83		0.3817				-0.4329		
6	3-Hexenyl acetate	9.21				0.1606				
7	2-Ethyl-1-Hexanol	9.77		0.1002						
8	2-Octenal	10.53		0.4261				-0.1233		
9	Heptanoic acid	11.02				-0.2253		-0.2579		
10	Nonanal	11.73		0.2657						
11	6-Undecanone	12.42		0.4638						
12	2-Ethyl-1-hexyl acetate	12.91				0.1030				-0.4635
13	(E)-2-Nonenal	13.13		0.2068						
14	Benzoic acid	13.25				0.2638				
15	(-)-Borneol	13.31		0.2346						
16	Octanoic acid	13.46						-0.2300		-0.2633
17	o-Methylacetophenone	13.77								0.3953
18	(+)-Alpha-terpineol	13.92	0.1046							
19	Methyl salicylate	14.02	-0.3252							
20	Decanal	14.27		0.3488						
21	2,2,3,4-Tetramethyl-3-cyclopenten-1-one	14.63								0.2584
22	Benzothiazole	14.73			-0.3254			0.5426		-0.1314
23	(+)-Carvone	15.22			-0.1921					
24	2-Decenal	15.60		0.3276						
25	Nonanoic acid	15.77				-0.1969		-0.3233		
26	3,7-Dimethyldecane	16.00							0.2923	
27	Undecanal	16.65		0.2242			0.2104			
28	7-Methyl-1-undecene	16.80	0.2562						0.2180	
29	5-Isobutylnonane	17.05							0.1438	
30	1-(2-Hydroxy-1-Methylethyl)-2,2-Dimethylpropyl 2-Methylpropanoato	17.68					-0.2805			
31	1,3,4-Eugenol	17.83				-0.5456				
32	2-Undecenal	17.95						-0.4846		
33	3-Hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate	18.15					-0.2676			
34	2-Bromooctane	18.59	0.1921		-0.1080			0.1848		
35	1, 4-Diacetylbenzene	19.45			-0.2903					
36	Methyl benzoate	19.57				0.1004				-0.1623
37	(E)-beta-Farnesene	19.91							-0.3849	
38	4,6-Dimethyldodecane	20.38			-0.3776		-0.3711			
39	2-Methyldodecane	20.68	0.4094				-0.2537		0.3426	
40	2,6,11-Trimethyldodecane	21.59	0.5309		-0.2203		-0.5460		0.3494	
41	1-Phenyl-1-butanone	22.19					-0.2043			
42	Heptadecane	24.80	0.5701				-0.5114		0.3541	

Table S6. Volatile compounds (with their coefficients) selected from the first two components of each sPLS-DAs of volatile metabolic profiles *T. pratense* plants after 24, 48, 72 and 96 hours of infestation with different pea aphid clones and uninfested plants (Figure 14). volatiles are sorted by their retention time (RT). For each component, only the loadings with the largest coefficient are presented. (+) and (-) next to the loadings indicate if they are positive and negative more abundant.

			24 hours		48 hours		72 hours		96 hours	
No	**Compound name**	**RT**	**comp 1**	**comp 2**	**comp 1**	**comp 2**	**comp 1**	**comp 2**	**comp 1**	**comp 2**
1	n-Hexanal	4.00				0.2745				
2	3-Hexenal	5.18				0.3266				-0.6600
3	3-Hexen-1-ol	5.26				-0.3654				0.2701
4	3-Methyl-2-cyclohexen-1-one	10.44	-0.5081							
5	1-Octanol	10.88		-0.3744				-0.3311		
6	Nonanal	11.74		-0.1497				-0.4776		
7	3-Methyl-6-hepten-1-ol	12.77			-0.2504			-0.3658		
8	2-Ethyl-1-hexyl acetate	12.92	-0.3772				-0.2692			
9	Octanoic acid	13.46		-0.4735				-0.4592		0.1982
10	(+)-Alpha-terpineol	13.93	-0.2519				-0.4492			
11	Decanal	14.27		-0.1687				-0.2778		
12	Ethylene glycol monophenyl ether	14.64		-0.3885						
13	2,2,3,4-Tetramethyl-3-cyclopenten-1-one	14.65					-0.1601			
14	Benzothiazole	14.73	-0.1763	-0.3102	-0.2896			-0.4122	0.4110	
15	2-Ethyl-1-hexyl propionate	15.08	-0.3434				-0.5173			
16	(+)-Carvone	15.21	-0.3805					-0.2409		
17	2-Decenal	15.62							-0.1901	
18	Nonanoic acid	15.76		-0.5698					0.1168	
19	1-Decanol	15.85			-0.4510		-0.1617			
20	Undecanal	16.67								0.1423
21	2-Ethyl hexyl butyrate	17.03					-0.2026			
22	1-(2-Hydroxy-1-Methylethyl)-2,2-Dimethylpropyl 2-Methylpropanoato	17.68	-0.1987		-0.4311		-0.3017		-0.3828	
23	1,3,4-Eugenol	17.82				0.6315				
24	3-Hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate	18.15			-0.4377		-0.2945		-0.4736	
25	2-Bromooctano	18.59	-0.3766		-0.3576		-0.4285			-0.3570
26	Vanillin	18.75								-0.3441
27	Dodecanal	18.90			-0.1777					0.1518
28	(E)-geranyl acetone	19.83				0.1972				
29	1-Dodecanol	20.26			-0.3163				-0.4839	
30	4,6-Dimethyldodecane	20.38	-0.2430							
31	1,3,4-Trimethyl-3-cyclohexene-1-carbaldehyde	20.56							-0.4180	
32	2-Methyldodecane	20.70								-0.3088
33	2,6,11-Trimethyldodecane	21.60				0.48647				-0.1059
34	Heptadecane	24.82								-0.2378

Table S7. Volatile compounds (with their coefficients) selected from the first two components of each sPLS-DAs of volatile metabolic profiles *P. sativum* plants after 24, 48, 72 and 96 hours of infestation with different pea aphid clones and uninfested plants (Figure 15). volatiles are sorted by their retention time (RT). For each component, only the loadings with the largest coefficient are presented. (+) and (-) next to the loadings indicate if they are positive and negative more abundant.

			24 hours		48 hours		72 hours		96 hours	
No.	**Compound name**	**RT**	**comp 1**	**comp 2**	**comp 1**	**comp 2**	**comp 1**	**comp 2**	**comp 1**	**comp 2**
1	(E)-2-Heptenal	7.86				0.1139			-0.5518	-0.1263
2	2-Ethyl-1-Hexanol	9.80			0.4688					
3	3-Methyl-2-cyclohexen-1-one	10.45	-0.1308							
4	1-Octanol	10.90		-0.2547						
5	Nonanal	11.75		-0.3618					-0.5070	
6	6-Undecanone	12.45				0.1455				-0.5206
7	2-Ethyl-1-hexyl acetate	12.94		-0.3332	0.2888					
8	(E)-2-Nonenal	13.15		-0.5279					-0.4516	
9	Octanoic acid	13.48		-0.2732						
10	Terpinen-4-ol	13.62			0.4866	-0.1145				
11	(+)-Alpha-terpineol	13.94	-0.2083		0.2668			0.3828		-0.2006
12	Methyl salicylate	14.04				0.4693				
13	Decanal	14.28		-0.4102					-0.3647	
14	2-Butyl-1-octanol	14.49	0.7081							
15	2,2,3,4-Tetramethyl-3-cyclopenten-1-one	14.66			0.1345					
16	Benzothiazole	14.75		-0.1998		0.2354	-0.1854			
17	2-Ethyl-1-hexyl propionate	15.10			0.3934					
18	2-Decenal	15.62							-0.2009	
19	Nonanoic acid	15.78		-0.3020				-0.4069		
20	1-Decanol	15.87					-0.4216			-0.5760
21	(3Z)-4,8-Dimethyl-3,7-nonadien-2-one	15.95			0.1318		-0.3569			-0.5241
22	7-Methyl-1-undecene	16.82	-0.1860							
23	1-(2-Hydroxy-1-Methylethyl)-2,2-Dimethylpropyl 2-Methylpropanoato	17.70					-0.3504			
24	1,3,4-Eugenol	17.83	0.5549							
25	2-Undecenal	17.93							-0.2355	
26	3-Hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate	18.18					-0.4893			
27	2-Bromooctano	18.61			0.4336		-0.5012			
28	Vanillin	18.76						-0.4357		
29	Dodecanal	18.91								-0.1513
30	Ethylene glycol monobenzoate	19.13								0.1922
31	1, 4-Diacetylbenzene	19.47		-0.1848				-0.5405		
32	(E)-geranyl acetone	19.84						-0.1369		
33	(E)-.beta.-Farnesene	19.94				0.8143				
34	1-Dodecanol	20.29					-0.1872			
35	4,6-Dimethyldodecane	20.41						-0.1988		
36	(+)-Beta-selinene	20.63						-0.1806		
37	2-Methyldodecane	20.70	-0.2132							
38	Tetradecanal	23.04						-0.1743		
39	n-Tetradecan-1-ol	24.23	-0.2149					-0.2901		

Table S8. Volatile compounds (with their coefficients) selected from the first two components of each sPLS-DAs of volatile metabolic profiles *V. faba* plants after 24, 48, 72 and 96 hours of infestation with different pea aphid clones and uninfested plants (Figure 16). volatiles are sorted by their retention time (RT). For each component, only the loadings with the largest coefficient are presented. (+) and (-) next to the loadings indicate if they are positive and negative more abundant.

			24 hours		48 hours		72 hours		96 hours	
No	Compound name	RT	comp 1	comp 2	comp 1	comp 2	comp 1	comp 2	comp 1	comp 2
1	2-ethyl-1-Hexanol	9.78	0.3857		0.3624					
2	6-Undecanone	12.41			0.3515					
3	(+)-Camphor	12.75			0.2079				0.3911	
4	2-Ethyl-1-hexyl acetate	12.92	0.3055		0.3399			0.5273	0.3325	
5	(-)-Borneol	13.32	0.3084		0.4915				0.3317	
6	Octanoic acid	13.47		0.4690	-0.1788				-0.3167	-0.2419
7	Terpinen-4-ol	13.60	0.4309		0.4007			0.3934	0.4061	
8	o-Methylacetophenone	13.79	0.2731							
9	(+)-Alpha-terpineol	13.92	0.2952		0.3604					
10	2,2,3,4-Tetramethyl-3-cyclopenten-1-one	14.59								-0.7480
11	Benzothiazole	14.73		0.5438						
12	2-Ethyl-1-hexyl propionate	15.08	0.3849					0.3517	0.2376	
13	2-Decenal	15.58					0.2273			
14	Hexyl benzoate	15.82			0.1403					
15	3,7-Dimethyldecane	16.01		0.1312						
16	3,4,5,6-Tetramethyloctane	16.21		0.5412						
17	7-Methyl-1-undecene	16.81		0.1998						
18	Beta-citronellyl acetate	16.87	0.3667							
19	2-Ethyl hexyl butyrate	17.02	0.1771							
20	5-Isobutylnonane	17.06		0.1876						
21	1-(2-Hydroxy-1-Methylethyl)-2,2-Dimethylpropyl 2-Methylpropanoato	17.67		0.1330			0.2712			
22	2-Bromooctano	18.58				-0.16062	0.7306			
23	Dodecanal	18.84								-0.3810
24	Ethylene glycol monobenzoate	19.13				-0.5053				
25	(Z)-beta-Caryophyllene	19.18								-0.4102
26	1, 4-Diacetylbenzene	19.44						-0.3181	-0.4375	
27	Methyl benzoate	19.67				-0.50763	0.3794			
28	Cuminone	19.78							-0.3105	
29	(E)-beta-Farnesene	19.82								-0.1067
30	4,6-Dimethyldodecane	20.38		0.2326						
31	Tetradecane	20.57						-0.4206		
32	2-Methyldodecane	20.66						-0.3162		
33	Tridecanal	20.96							0.1277	
34	2,6,11-Trimethyldodecane	21.59		0.1589				-0.2061		
35	Benzoic acid, hept-3-yl ester	22.16				-0.43305				-0.1505
36	Benzoyl methyl ketone	22.39				-0.40921				
37	Tetradecanal	22.98					0.3982			-0.1081
38	n-Tetradecan-1-ol	24.15								-0.1419
39	2-Dodecanone	24.59				-0.29415	0.1519			
40	Heptadecane	24.78						-0.1223		

Table S9: Statistical values for the analysis of GLV in *M. sativa* plants according to aphid clone, time of aphid infestation, and the interaction between aphid clone and time of aphid infestation.

Green leaf volatile	Factor	Variance	L-ratio	P-value
(Z)-3-Hexenal	interaction	treat	19.720	**0.02**
	time		14.854	**0.002**
	clone		40.904	**<0.001**
(Z)-3-Hexen-1-ol	interaction	race	52.289	**< 0.001**
	time		35.066	**<0.001**
	clone		17.385	**0.0006**
(Z)-3-Hexen-1-ol acetate	interaction	treat	22.958	**0.0063**
	time		35.642	**<0.001**
	clone		23.112	**<.0001**

8 Selbständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig und unter Verwendung der angegebenen Hilfsmittel, persönlichen Mitteilungen und Quellen angefertigt habe.

Jena, den Carlos Fernando Sanchez-Arcos

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